INDICES OF, AND PROTECTIVE MECHANISMS AGAINST, LIPID PEROXIDATION IN BLOOD AND CEREBROSPINAL FLUID IN MULTIPLE SCLEROSIS

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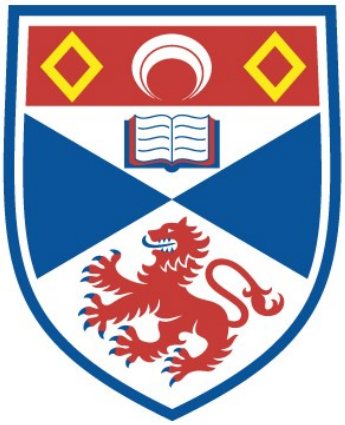

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LIPID PRODUCTION AND COMPOSITION IN HAPLOID AND DIPLOID STRAINS OF *ASPERGILLUS NIDULANS*

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by

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SUMMARY

Six auxotrophic mutants of *A. nidulans* were crossed in a dialell cross system to obtain heterokaryous and heterozygous diploids.

In order to ascertain their lipid accumulation ability, some of these mutants and diploids were tested in a minimal medium (with 3% glucose + 0.6% $NaNO_3$), either in a shaker or in an incubator without agitation. Both, the mutants and diploids, exhibited only 4.6% lipid, on a dry weight basis.

With the aim of optimising culture conditions for lipid accumulation, a wild type was cultivated in a range of different media and cultural conditions. The best yield (about 24%), was achieved in a modified minimal medium (MM + 12% glucose + 0.1% $NaNO_3$), with a vortex stirrer device.

The lipid composition of wild type *16* grown in a fermenter was determined. The results obtained from cells grown in two different media and using two extraction methods were compared.

Fractionation of the total lipid on a Florisil column showed that this strain is composed of 86% neutral lipid, 7% glycolipid and 7% phospholipid, after isopropanol (IP) extraction, whilst chloroform-methanol (CM) extraction gave 75% neutral lipid, 8% glycolipid and 17% phospholipid.

A further fractionation on hydrated Florisil showed that CM extracted sterols (both free and esterified) more efficiently than IP. Therefore, CM was considered a better extraction method, particularly for protein-bound lipids.

The separation of the neutral lipid fraction into sub-classes also showed that the enhanced lipid content achieved in modified minimal medium, compared with a previously reported medium, was accounted for mainly by an increase, not in the triglycerides as was expected, but in the amount of sterols.

TLC analysis of glycolipid and phospholipid from IP and CM extraction demonstrated two major glycolipid components (monoglycosyl and diglycosyl

diglycerides) and that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the principal phospholipids with lesser amounts of phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, cardiolipin and phosphatidic acid (PA) after CM extraction, whilst after IP extraction only PC, PE and PA were found. Another significant difference between the two extraction methods is the large amount of PA found after CM extraction, but not after IP, showing that, almost certainly, phospholipase D activity had occurred during the process of extraction and/or storage of the lipid. It was also found that the principal phospholipid attacked by the enzyme was PC.

The fatty acid composition was determined by GLC. The major fatty acids found in the total lipid were: 16:0 = 21%; 17:0 = 5%; 18:0 = 18%; 18:1 = 20%; 18:2 = 35%.

Each lipid class showed a different and distinctive fatty acid composition, exhibiting variation with the growth medium and extraction method used. Of particular interest was the sterol ester fraction which contained margarinic acid (17:0) as its only fatty acid.

Declaration

I hereby declare that this thesis has been composed by myself, and that it is a record of work which has been done by myself. None of the work has been accepted in any previous application for a degree. Any other sources of information have been acknowledged.

Signed

Regina Teresa Rosim Monteiro

Statement

I, Regina Teresa Rosim Monteiro, was admitted as a research student of the University of St. Andrews in October 1981 in accordance with Ordinance General No. 12 and the Resolution of the University Court, 1967, No. 1. The thesis was completed in September 1984.

Certificate

I hereby declare that Regina T.R. Monteiro has been engaged upon research from October 1981 onwards to prepare the accompanying thesis for the degree of Doctor of Philosophy.

Signed

Dr. M.I.S. Hunter

St. Andrews

September 1984

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CONTENTS

I. INTRODUCTION

The exploitation of fungi by man for the production of commercially important products is not a recent phenomenon. The use of yeast to produce alcoholic beverages and to leaven bread, for example, dates back to biblical times.

Microorganisms have long been recognised as capable of producing large quantities of lipids. The first fungus to have more than theoretical interest shown in its lipid content was *Endomyces vernalis*. The acute shortage of fat towards the end of the 1914-1918 war, in Germany, led to a large scale project for the production of fat as a source of energy (fuel) and food (Hesse, 1949). In this project they reached yields of up to 25 percent of the dry weight of *E. vernalis*. Difficulties of surface culture, high costs and high labour demand resulted in the abandonment of this project after the war. However, the attempt to operate on a commercial scale was again taken up before and during the second world war by two German industrial groups using, this time, submerged cultures of *Torula* and *Fusarium* to act as fat formers. Apart from the technical success, the process was once more considered uneconomic (Woodbine, 1959) after the war.

The increase in world population and in industrialization has led to a search for new sources of energy, food supply and ways of eradicating pollution in our environment. Many of these problems have been solved by microbial technology, new microbial cultivation techniques have been developed in the last 40 years for the production of antibiotics, protein (single-cell-protein), citric acid, etc. on a large scale. These cultivation techniques have been used in countries with very little fossil fuel reserves to obtain fuel by fermenting raw or partially refined materials to produce biogas or liquid fuel, e.g. fermenting sugar cane or cassava starch to produce ethanol.

All these newly developed techniques of microbial cultivation

combined with the scarcity and rising prices of their counterparts increase the possibility of obtaining oils and fats from a microbial source on a commercially viable scale (Ratledge, 1982).

The possibilities for the use of microbial lipid by my country (Brazil) are promising, as the government is determined to build a chemical industry based on biomass and so become independent of foreign imports that have to be paid for in hard currency (Goldemberg, 1978; Nawawy, 1982).

There are enormous advantages to be gained by using microorganisms as producers of important commercial products. For example;

(i) rapid growth rate - they can double their number in a very short time: 0.3-2.0 hours for bacteria, 1-3 hours for yeast, 2-6 hours for algae and 4-12 hours for filamentous fungi;

(ii) a relatively small area is required - according to Nawawy (1982) 2 million tonnes of microbial protein (single cell protein) can be produced in 2,000 fermenters, each with a capacity of 200 cubic metres. 2,000 fermenters can be accommodated within one hectare of land, while the cultivation of soya beans to produce the same amount of protein, would occupy 40 million hectares of land: almost an impossibility;

(iii) a better control of the cultural conditions, and thereafter of the product, is possible;

(iv) there are possibilities for greatly improving product yield through strain selection by mutation and recombination and by optimization of growth conditions.

The oil and fats used by man come from a variety of sources, both animal and vegetable, and are destined for foodstuffs such as margarine, butter, lard, cooking and salad oils, or for other manufacturing purposes to produce cosmetics, soaps, shampoos, detergents, paints, waxes and polishes, etc.

The lipid from a microbial source should be judged in accordance with its intended purpose (Ratledge, 1978). One may be interested in either the intact triglyceride or in the fatty acids derived from the acylglycerols. If the lipid is to be used for a technological purpose such as the manufacture of soaps, shampoos, lubricating greases and oils, etc., the total fatty acid composition should be analysed. If, on the other hand, the lipid is to be used for foodstuffs, it is the triglyceride fraction which is the most important and should be separated from unwanted fractions including phospholipids and sterols. In the latter case, toxicological trials will be required (Ratledge, 1978). The toxicological problems should not be exaggerated as the lipid will be an extracted product (not the whole organism) and will therefore be refined and purified by the same techniques used for existing plant oils.

The lipid content and fatty acid composition of several micro-organisms have been investigated by a number of researchers. A complete review by Woodbine (1959) covers the work up to late 1950's and more recent reviews can be found by Weete (1980) and Ratledge (1982).

Microorganisms with the potential for lipid accumulation are mainly algae, yeasts and fungi. The prokaryotes are considered unsuitable (Ratledge, 1982), largely because of their lipid composition, which tends to be complex, or because of difficulties of extraction and, therefore, they could not be used as an alternative source to plant oils or animal fats.

Studies on algae, as lipid sources, began in the 1940's and in recent years considerable attention has been paid to their lipid composition.

The lipid composition of algae can be greatly altered by cultivation conditions. In some instances, certain algae produce more than 50 percent of their dry biomass as specialized lipids. An example of this is *Botryococcus braunii*, a fresh water organism , that

accumulates hydrocarbons up to 86 percent of its dry weight (see Dubinsky *et al.*, 1978). The feasibility of using the oil from *Botryococcus braunii* as a transport fuel has been studied by Hillen *et al.* (1982), who found that, after the hydrocracking procedure, it was suitable to use as petrol and aviation turbine fuel.

Another attraction of algal lipids is that they are rich in dietary essential polyunsaturated fatty acids and so are potentially important for human and animal nutrition (Ratledge, 1982).

Yeasts and moulds are the microorganisms most extensively studied for their lipid producing ability under laboratory conditions. Several species of yeast can produce 30 to 70 percent lipid. Some of these include the *Rhodotorula* and *Lipomyces* species.

Yeasts, rather than moulds, have been chosen for growth under fermenter and continuous culture conditions, as there are serious limitations in the process when filamentous fungi are grown, e.g. they accumulate most of their growth on surfaces inside the fermenters and within pipes and valves, rather than in the medium. Various mechanical techniques have been used to minimise these problems and new fermenter designs have been created to grow filamentous fungi (Bull and Bushell, 1976). Another way of solving this is selection of strains which have a low affinity for wall growth (Solomons, 1980). With these recent developments, therefore, the time may be ripe for reinvestigation of the filamentous fungi as lipid producers.

The principal moulds capable of producing a high quantity of lipids are species of *Claviceps*, *Penicillium*, *Aspergillus*, *Mucor*, *Fusarium* and *Phycomyces* (Woodbine, 1959).

Most of the moulds are slower growing than the yeasts, but the former can produce oils containing higher concentrations of polyunsaturated fatty acids (Ratledge, 1978).

The organisms chosen for this study were several strains of

Aspergillus nidulans. This species has the advantage of a great deal of background knowledge of its genetics. Also mutants suitable for crossing and diploid formation were easily available. In addition, several reports had already indicated the ability of this mould to accumulate considerable amounts of lipids.

In view of the number of species of microorganisms, relatively few have the ability to accumulate lipids and even in the majority of these cases special growth conditions are required. Selection of a suitable microorganism is therefore essential for any potential commercial process.

Many techniques have been employed, with great success, using industrial stains to improve yields of other microbial metabolites, such as penicillin, cephalosporin, citric acid, kojic acid and amylases. There are basically three ways (Johnston, 1975) in which strains can be improved:

(i) by selection alone;
(ii) by mutation followed by selection, and
(iii) by recombination and selection.

The technique of selection alone is a fastidious process that involves the isolation and purification of a large number of cultures of interest, usually from the natural environment, and subsequent selection in order to find the few strains capable of highest productivity. Many examples of surveys of several mould species, by different researchers looking for potential lipid accumulating strains, can be found in Woodbine (1959).

Mutagenesis followed by selection is a conventional method used in both industrial and applied microbiological laboratories, particularly to improve the yields of other microbial products such as antibiotics, organic acids, amino-acids and enzymes. Much of the existing literature, dating back to 1948-1958, deals with the selection of penicillin-producing

strains. In most cases the mutants were obtained by using ultraviolet light, x-rays, nitrogen mustards and ethylenimine.

In the last three decades, many refinements have been introduced into this technique, making it a very effective method of improving fermentation production. Mutation programs are applied most often to the current industrial strains which are already highly stable, productive and well adapted to the fermentation process.

Unfortunately, the phenomenon of mutation is random and not directable, so that mutation as applied to strain development is largely empirical. Consequently, a method for the rapid surveying and evaluation of new variants represents the key to a successful strain development program.

Mutagens are frequently divided into two classes: physical (radiation) and chemical. Apart from technical problems such as ease of use, perhaps the most important consideration in choosing between mutagens is whether they will give rise to different spectra of mutant types. Since, frequently, little is known in advance of what kind of mutation will be produced, it is important to choose a mutagen with as wide a spectrum as possible, which will affect all areas of the genome with equal frequency, so that the screen will not fail due to lack of mutation at the necessary site. Rowlands (1983) recommends the use of ultraviolet light, ethyl methane sulphonate (EMS) and nitrosomethylguanidine (NTG).

Industrially important mutants can be subdivided into distinct classes whose properties facilitate their subsequent selection. Examples are: morphological, auxotrophs, resistants and revertants.

Morphological mutants are usually selected from spore suspensions exposed to small or moderate doses of a mutagen and may vary in morphology or colour.

Auxotrophic mutants are recognized by their lack of growth on a medium (minimal medium) containing the minimum nutrients essential for the growth

of the wild type, but these mutants will grow if one or more specific substances are added to the medium.

A characteristic peculiarity of auxotrophic mutants is their ability to accumulate intermediate products of a pathway preceding a particular genetically blocked reaction, the intermediate products often being able to undergo further biochemical transformations.

Mutations producing drug resistance have been selected in a number of fungi by plating cells at high densities on a medium containing a high enough concentration of the drug in question to inhibit wild type growth. Mutants resistant to analogues of normal metabolites are particularly interesting since they may show modifications of normal metabolic regulatory mechanisms. In the presence of an antimetabolite (i.e. substance similar in chemical structure to the natural metabolite) in the medium of growth, resistant mutants will grow up, showing that there is a derepression (or lack of feed-back inhibition) of the corresponding enzyme system.

Other kinds of mutant that have been used, mainly to increase the efficiency of antibiotic production, are revertant mutants. There are many examples of revertants of a non producer parent which show greater activity than the original grandparents culture (Elander and Chang, 1979).

The third way of improving fermentation using microorganisms is by recombination and selection. The potential of the recombination and selection technique, for developing improved strains, has been apparent since the elucidation of the parasexual process in fungi by Pontecorvo *et al.* (1953) and Pontecorvo and Sermonti (1953).

The contribution of breeding to strain improvement may be considered at three levels in the fungal life cycle: cytoplasmic interactions (heterokaryons), sexual crosses and parasexual crosses.

The majority of work done on the breeding of industrial strains has

been via the parasexual cycle. The sexual cycle is of minor importance in industrial fungal genetics as it does not appear to be present in any of the species used for most fermentation products, such as penicillin, cephalosporin and citric acid. *Aspergillus nidulans* which has both sexual and parasexual cycles is often used to elucidate the genetics of antibiotic production (Rowlands, 1983).

The use of planned breeding in filamentous fungi, as one attempt at improving the ability to synthesize economically important fermentation products, has been practised for penicillins (Caglioti and Sermonti, 1956; Pathak and Elander, 1971) kojic acids (Ikeda *et al.*, 1957) and citric acid (Ilczuk, 1971, Baracho and Monteiro, 1980), but no efforts in this direction have been applied to lipids.

The potential of parasexual crosses has been extended by the relatively new technique of protoplast fusion. Using this technique, problems of compatibility barriers between species may be overcome. Genetic analysis and manipulation can then be carried out to create strains having features of both parents (Peberdy, 1980).

The application of genetic engineering techniques has not been reported in industrial filamentous fungi. However, some evidence exists that this technique might have a great impact on fungal biotechnology (Rowlands, 1983).

Reviews with more detailed information on methods of selection and hybridization in filamentous fungi to improve industrial production can be found in Alikhanian (1970), Johnston (1975), Elander and Chang (1979) and Rowlands (1983).

The genetic dependence of lipid accumulation can be recognised from several examples where either the ability to accumulate lipid by a given strain, even in unchanged conditions, is lost (Ratledge, 1982) or where there is considerable variation in lipid content between different isolates of the same species cultured under identical conditions.

For example, two strains of *Fusarium lini* grown on the same medium differ by 100 percent in their lipid content (Weete, 1980).

Besides the choice of a suitable microorganism which has the potential for lipid accumulation, certain special cultural conditions are necessary to ensure high lipid production. Lindner (see Hesse, 1949) using *E. vernalis* to produce fat during the First World War, was the first to recognize that an abundant supply of carbohydrate was necessary for lipid accumulation. He also pointed out that inocula should consist of freshly grown cultures and that oxygen should be supplied. These points have been confirmed by subsequent studies (see Ratledge, 1982). In addition, other conditions have also been recognized as pre-requisites for lipid accumulation, such as growth on a limiting amount of a nutrient, other than carbon, usually nitrogen. The growth limiting factor is allowed to become exhausted so that any excess carbon will continue to be taken up and, in an oleaginous organism, will be converted into lipid.

Lack of essential nutrients such as nitrogen, potassium, or phosphorus, make impossible synthesis of new RNA, protein and other compounds necessary to continue growth. Oleaginous organisms are able to continue to take up the carbon from the medium and convert it into lipid. Ratledge (1982), emphasised that there is no increase in the actual rate of lipid synthesis, lipid accumulation occurs because other metabolic processes cease.

A two-stage pattern of growth and lipid accumulation can thus be recognized in an oleaginous microorganism growing in batch culture: in the first stage, the cells grow at their maximum rate until one (or all) nutrients, except glucose, are exhausted and only then does the stage of lipid accumulation begin.

Borrow *et al.* (1961), studied the metabolism of *Gibberella fujikuroi* (*Fusarium moniliforme*) when grown in a stirred tank fermenter with selective exhaustion of nutrients. The growth media were prepared, initially, with variable concentrations of glucose, nitrogen, phosphorus

and magnesium, such that, at a desired culture biomass, one nutrient was exhausted and the other nutrients would be exhausted in specific sequences thereafter. Five distinct growth phases were found:

(i) balanced phase: where exponential growth occurred until the first nutrient was almost exhausted

(ii) transition phase: the growth and uptake rates were reduced with phosphorus and magnesium exhaustion.

(iii) storage phase: with nitrogen exhaustion the growth ceased and in the presence of glucose, lipid and carbohydrate synthesis continued

(iv) maintenance phase: no nutrients were taken up at this stage with the exception of glucose, if present. Depletion of glucose in the medium in nitrogen-limited cultures led to endogenous lipid metabolism

(v) terminal phase: period of hyphal breakdown.

However, some oleaginous organisms do not exhibit this pattern of lipid accumulation, as they always seem to have a high content or accumulate lipid during the growth phase, even when no nutrient is limiting. Such is the case in the yeast *Cryptococcus terricolus*, in which high amounts of lipid are found independent of the quality and quantities of nitrogen source, and of the phase of growth (Pedersen, 1961).

Studies of lipid formation by *A. nidulans* were initiated with the work of Woodbine *et al.* (1951), who studied the fat potential of 43 strains of moulds representing 10 genera on 5 different media all with glucose as carbon source. The best results for lipid accumulation were from *A. nidulans* (25 percent, w/w), *Aspergillus flavipes* (40 percent, w/w) and *Fusarium lini* (35 percent, w/w) and the medium used by Prill *et al.* (1935) was the best of those tried.

In similar experimental conditions, Murray *et al.* (1953) examined

the behaviour of the same 43 strains in four different media, all with sucrose as carbon source. The highest lipid contents (dry weight basis) were 35 percent with *Penicillium soppi*, 28 percent with *F. lini* and 26 percent with *A. nidulans*.

A more detailed study was carried out by Gregory and Woodbine (1953) on *A. nidulans*, *Penicillium spinulosum* and *Penicillium javanicum*. This included varying the pH, temperature, incubation period, and nitrogen and sugar concentration. They found that the effect of halving the glucose while keeping the ammonium nitrate concentration constant (in Prill *et al.* (1935) medium), lowered the yield of lipid and the amount of sugar used in *A. nidulans*. Keeping the same C:N ratio, but raising the glucose concentration from 10 to 20 percent, did not improve the efficiency of convertion of sugar to lipid, but reduced it. They concluded that a high nitrogen:carbon ratio would favour the production of protein and a low nitrogen:carbon ratio would favour that of lipid.

The effects of diverse mineral supplements on these three moulds were reported by Garrido and Walker (1953a) and Garrido *et al.* (1953) who showed that lipid formation in moulds could be enhanced to a considerable degree by adjusting the quantities and proportions of sodium, potassium, magnesium, sulphate and phosphate ions. In a medium of (per 100 ml) 500 mg magnesium sulphate for all 3 moulds, but 155 mg phosphorus and 10 mg potassium for *A. nidulans*; 15 mg phosphorus and 195 mg potassium for *P. javanicum*; and 7 mg phosphorus and 195 mg potassium for *P. spinulosum*, lipid contents were 50%, 24% and 25%, respectively.

Garrido and Walker (1953b) studied the behaviour of *A. nidulans* when grown in glaxo flasks, showing that the biochemical activities of moulds in surface growth experiments are influenced by physical factors consequent on the type of vessel used for cultivation and, in particular, by the ratio of surface area/volume.

A. nidulans, *P. javanicum* and *P. spinulosum* were also used by Gad and Walker (1954) in a study of the formation of lipid from sucrose;

and Garrido and Walker (1956) compared the efficiency for lipid production of glucose, arabinose, xylose, ribose, galactose, maltose, lactose, inulin and starch. Xylose was found to be the next best carbon source to glucose. These workers also studied the effect of six nitrogen sources. Ammonium nitrate and sodium nitrate showed the best results after 6-8 days of growth at 25°C.

Continuing the studies with these three moulds, Garrido *et al.* (1958) determined the optimum concentrations of sodium dihydrogen phosphate, magnesium sulphate and potassium sulphate, in shaken culture. Under these conditions, the highest yields of lipid as g/l of culture broth were: *A. nidulans*, 8.9; *P. javanicum*, 3.7, and *P. spinulosum*, 9.1.

In a stainless steel fermenter of the Waldhof type, Garrido and Walker (1958) grew these three moulds in the same medium as that previously used for shaken culture. The yields of lipid (g/l) were as follows: *A. nidulans*, 4.4; *P. javanicum*, 3.6, and in a different medium, *P. spinulosum* produced 11.4. According to these figures therefore the lipid accumulation ability of *A. nidulans* in the fermenter was lower than in shaken cultures. This lower value was attributed to the fact that there was a tendency for the new mycelial growth to adhere to the wall of the fermenter, forming dense sticky aggregates which probably received insufficient oxygen, with consequent diminution in the lipid yield.

In 1959, Gad *et al.* described the cultivation of the moulds *A. nidulans* and *Penicillium soppi* in surface and in shaken culture, with cane and beet molasses as substrates. Satisfactory yields of lipid on a cane molasses medium supplemented with salts (up to 16 g/l) and, on an unsupplemented beet molasses medium (up to 11.8 g/l) were obtained with *A. nidulans* in static culture, but in shaken cultures prepared with molasses media, this mould produced little lipid. In static and in shaken cultures, prepared with sucrose-salts media *P. soppi* produced 21.6 and 8.1 g/l, respectively.

Naguib (1959) grew *A. nidulans* on four different sugar sources

(glucose, sucrose, fructose, or maltose) and Naguib and Saddik (1960) also grew the same mould on three different nitrogen sources, in surface culture. Growth, sugar and nitrogen uptake and synthesis of carbohydrates, proteins and lipid were also followed over an incubation period of 20 days. It was found that glucose was more readily metabolized than the other sugars, giving a heavy felt with the highest lipid content in a shorter time and that asparagine was far superior to nitrate or ammonium as a nitrogen source for lipid formation. Protein and carbohydrate contents were higher in nitrate than in asparagine or ammonium fed mycelial felts.

The ability of *A. nidulans* to use ammonium salts as nitrogen source was investigated by Naguib and Saddik (1961). Three different ammonium salts, namely: chloride, carbonate and nitrate were evaluated by comparison with growth in sodium nitrate. Ammonium carbonate gave the highest lipid content.

Mycelial mats of *A. nidulans* were grown in a medium made from sweet potatoes by Naguib and Adel (1962) and contained 15 percent (w/v) lipid.

These studies with *A. nidulans* were carried out at Manchester University using a single wild type strain. This strain, selected by Woodbine *et al.* (1951), exhibited good capacity for lipid accumulation, which could be improved by subsequent changes in medium and conditions of growth. *A. nidulans* was also shown to be suitable for growth in media of high salt concentration (so agro-industrial residues, such as molases, could be used as growth medium), when cultivated without agitation, but in submerged culture better yields were obtained in media with lower concentrations of salt (Garrido and Walker, 1953a).

The fatty acid composition of *A. nidulans* lipids, studied by Singh *et al.* (1955) using a low temperature crystallization technique for analysis, was found to be (percent): 14:0 = 0.7; 16:0 = 20.9; 16:1 = 1.3; 18:0 = 15.9; 18:1 = 40.3; 18:2 = 17.0; 18:3 = 0.2; 20.0 = 3.8.

Changes in the lipid composition of this mould, with the age of

the culture, were reported by Singh and Walker (1956a). Estimation of acid value, saponification equivalent, iodine value and unsaponifiable matter were performed.

It was concluded that the rate of formation of fat in surface culture is slow during the first week of incubation, but becomes very rapid during the eighth and ninth days, when the maximal development of the mould is reached. After the ninth day, the reserve of fat in the mould decreases in weight. In the early stages of fat synthesis, the fat is more acidic and contains a larger percentage of unsaponifiable substances than at any later period. Until stationary phase of the culture is attained, the amounts of both saturated and unsaturated component acids containue to increase, but in the subsequent phase of the culture, the weight of each of these components diminishes.

This experiment was designed to answer the question as to whether the unsaturated acids that are components of the fat of this organism were formed by dehydrogenation of saturated acids or some other mechanism. It was concluded that the results were in accord with some alternative mechanism.

The influence of pH of the medium on the lipid composition of *A. nidulans* was studied by Singh and Walker (1956b). The iodine value of the fatty acids increases with the pH of the medium, which is due to an increase in oleic acid with a corresponding decrease in the amount of saturated acids.

The same authors (1956c) found also that the lipid synthesized at 17°C was more unsaturated than at 25°C, but more unsaturation was also observed at 30°C than at 25°C, so that a simple relationship between growth temperature and degree of unsaturation was not found.

The effect of sucrose concentration (10-40 percent) was reported by Singh and Datt (1957), who could that the optimum sugar concentration for the maximum yields of mycelial mass and lipid formation was 20 percent and the lipid produced at this concentration exhibited the greatest degree

of saturation. The least amount of felt and fat are formed with 40 percent sugar and the fat produced at this concentration contains the smallest proportion of saturated acids, while the amounts of oleic and linoleic acids are higher than at lower sugar concentrations.

Studies on changes in the proportion of the component fatty acids of *A. nidulans* lipid at different levels of ammonium nitrate concentration (0.15-0.60 percent) in the medium were reported by Singh and Datt (1958). The production of lipid is maximal with 0.30 percent ammonium nitrate and the fat elaborated contains the largest proportion of saturated acids. The yield of the mycelial mass is highest at 0.60 percent, though the percentage of fat, at this concentration, is lower. The lipid produced at this N-source concentration contains the largest proportion of oleic and linoleic acid.

All these studies on the lipid composition of *A. nidulans* were again performed using the same wild type strain studied by the Manchester University group. From these experiments it can be deduced that this strain exhibits an early phase of growth during which there is a high rate of protein synthesis and slow rate of lipid synthesis. Later, protein synthesis slows down while lipid content increases. Also, the fatty acid composition was affected by changes in temperature, pH, sucrose and ammonium nitrate concentration and the incubation period.

More recently, ergosterol was identified as the major free sterol of *A. nidulans* by Shapiro and Gealt (1982). Lanosterol, a precursor of ergosterol was identified as a minor component of the free sterols, but was usually more abundant than ergosterol in the steryl ester fraction. Because of this, it was concluded that the esters serve as storage compounds for the membrane sterol precursors.

The content of sterol and phospholipid components of *A. nidulans*, grown in the presence and absence of fungicides, was determined by Craig and Pederby (1983). The two drugs studied were shown to affect both

the quality and composition of the lipid components studied. In the absence of drug the proportion of components were: sterol - 4.88% (w/w), phospholipids - 30.76% (w/w). The percentage of phospholipid classes (w/w) found were: phosphatidylcholine - 47%; phosphatidylethanolamine - 37.5%; phosphatidylinositol - 10.5% and phosphatidylserine - 4.0%.

Lipids extracted from living organisms, are normally complex mixtures that can be easily separated into neutral and polar lipid fractions. Common neutral lipids are triglycerides (or triacylgycerols), sterols and sterol esters. Predominant polar lipids are phospholipids and glycolipids.

The proportion of each fraction depends on the organism and cultural conditions.

Fatty acids are produced by all living organisms and potentially represent the most abundant class of lipids in nature. However, free fatty acids do not occur naturally to any great extent, but are mainly esterified as constituents of acyl glycerides (tri- di- and monoglycerides), phospholipids, glycolipids and sterol esters.

The lipids of prokaryotic micro-organisms have a large number and variety of fatty acids, many of which are uncommon, or unknown, in fungi and other eukaryotes (O'Leary, 1967). The greatest difference of all between prokaryotics and filamentous fungi and other eukaryotes is the fact that bacteria and other prokaryotic cells do not synthesize polyunsaturated (polyenoic) fatty acids (except some blue-green algae), whereas these fatty acids are ubiquitous in eukaryotic cells (Shaw, 1966).

Bacterial fatty acids usually consist of simple saturated and monoenoic components, but may also contain odd-numbered, branched chain and cyclopropane acids.

The major fatty acids of eukaryotic organisms contain an even number of carbon atoms in straight chains with a terminal carboxyl group and may be fully saturated or contain one, two or more (up to six) double

bonds, which generally have a cis configuration. The commonest fatty acids are straight chain even-numbered acids containing 14-20 carbon atoms, although all the possible odd and even numbered homologues with 2-30 or more carbon atoms have been found in nature. The saturated fatty acids, myristic (14:0), palmitic (16:0) and stearic (18:0), and the unsaturated fatty acids, oleic (18:1), linoleic (18:2) and linolenic (18:3), together account for the majority of the fatty acids of eukaryotic microorganisms.

Algal lipids usually contain a variety of fatty acids, which may vary considerably according to culture conditions. In general, the homologous series ranging from 14-22, characterized by a relatively large proportion of polyunsaturated C_{18}, C_{20} and C_{22} fatty acids are found (Weete, 1980).

The fatty acid composition of yeasts and moulds, in general, is as follows: 16:0 is the predominant saturated fatty acid, and 18:1 and 18:2 the predominant unsaturated fatty acids. The proportion of 18:2 being higher in moulds than in yeast, which generally possess 18:1 as the major unsaturated fatty acid (Whitworth and Ratledge, 1974).

Fatty acids are important as sources of energy in acyl lipids, and some with specific structures are required for growth, presumably as structural components of membrane lipids (Weete, 1980).

The degree of unsaturation of the fatty acids esterified to polar membrane lipids is well recognized as having a profound effect on the physical properties of the membrane (fluidity) and consequently on all aspects of membrane function (Moore, 1982).

The most abundant class in lipid extracts of fungi is usually triglycerides (Ratledge, 1982). The properties of the triglycerides are governed mainly by the constituent fatty acids, but partly also by which of the three possible positions the different fatty acids occupy on the glycerol molecule. Plant triacylglycerols tend to have very few saturated

fatty acids in the 2-position, whereas this is not true with triglycerides of animal origins. So plant oils will contain primarily SUS, SUU and UUU triglycerides (S = saturated; U = unsaturated fatty acid), there will be relatively few SSS, SSU or USU triglycerides, as found in animals. From the available evidence, yeast and mould triglycerides are similar to the plants oils, in this aspect (Ratledge, 1982).

The major commercial uses of triglycerides are in the manufacture of margarine, cooking and salad oils, with their quality very dependent upon their fatty acid composition and distribution.
Triglycerides are considered as reserve lipid and are believed to be stored in the form of droplets in the cytoplasm. Triglycerides are about two times more efficient, on a calorie/g basis, for metabolic energy, than either proteins or carbohydrates.

With very few exceptions, a very high content of triglycerides is present in the total lipid of yeast cells; values are frequently above 85 percent of the total lipid. Unfortunately, analysis of oils from filamentous fungi has been mainly on the relative proportions of fatty acids of the total lipid, although some work has been carried out on lipids fractionated into neutral and polar classes (see Ratledge, 1982). However, this tells us little about the proportion of the commercially important triglycerides compared with other neutral lipids such as sterols and sterol esters.

Mono and diglycerides are rarely present in more than trace amounts, as found in plants or animal tissues, where 1,2 diglyceride has a particular importance as the precursor of triglycerides and complex lipids (Christie, 1982).

Sterols are present in most eukaryotic microorganisms, except under anaerobic conditions when, in principle, sterols cannot be biosynthesised, since the introduction of the 3 β-hydroxyl group is a reaction requiring

molecular oxygen (Nes, 1977).

The most common sterol of yeast and moulds, is ergosterol and it is often accompanied by several other sterols in lower relative proportions.

The sterols occurring in fungi and their functions are the subject of recent reviews by Brennan *et al.* (1974), Elliot (1977), Weete (1980) and these are summarised below.

Sterols play an important role in the architecture of membranes and may also regulate the permeability by affecting internal viscosity and molecular motion of the lipids in the membranes. Sensitivity to polyene antibiotics has been related to membrane sterol content. They are also required by a number of fungi for sexual reproduction and can stimulate growth in aerated culture. Some fungi require sterols for optimum activity of certain enzymes such as ATPases (Weete, 1980).

The sterol content of a particular microorganism is highly variable and very sensitive to conditions of growth, specially the concentration as well as type of sugar used as carbon source. In general, high ergosterol content is associated with conditions allowing vigorous growth or under conditions of stress, such as very low or very high temperature, exposure to fungicides or other toxic substances.

In some organisms such as *Saccharomyces cerevisiae*, in cases where high levels of total lipid have been reported, almost half the lipid isolated from intracellular oil droplets was found to be sterol, either free or as sterol esters.

The potential commercial importance of sterols is exemplified by the exploitation by the pharmaceutical industry of ergosterol, as a precursor of other economically important sterols (Brennan *et al.*, 1974).

Sterol ester is another neutral lipid component, which is widely distributed in nature and is also reported to occur in fungi where there is little known about its function and location in the cell.

It seems possible that sterol esters serve as a source of sterol for synthesis of new membranes and are stored in small low-density vesicles

(Elliot, 1977; Rose, 1981).

The fatty acid composition of sterol esters reported in fungi, ranges in chain length between 14-18, esterified primarily to ergosterol (Wassef, 1977).

Phospholipids are considered the second major lipid component in fungi and have long been recognized, with glycolipids, as the major lipid components of membranes.

In general, the major phospholipids reported to occur in fungi are phosphatidylcholine (PC), phosphatidylethanolamine (PE) with phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL) occurring in lesser amounts.

Functions of phospholipids are mainly associated with the membranes, having a profound influence on cellular activities through control of membrane permeability and membrane-bound enzyme activities. Most of these systems have been studied in mammals, however, there are several examples of this type of interaction in fungi. For example, CL is known to be associated specifically with mitochondria in yeast and in *Aspergillus niger* (as in all other eukaryotes). PE has been implicated in lysine uptake by *Neurospora crassa*. PI seems to be particularly involved in membrane transport of phosphate and arsenate, and transport of protons and other cations across yeast membranes (for references, see review by Brennan and Lösel, 1978).

The effect of fungicides on the sterol and phospholipid composition of *A. nidulans* lipid fractions was studied by Craig and Pederby (1983). They found that both sterol and phospholipid composition (mainly PC) were affected by the two drugs studied.

Glycolipids are complex lipids that can also occur in fungi. Generally, glycolipids have not been widely studied in fungi, and relatively little is known about their occurrence in these organisms (Weete, 1980). Cerebrins have been regarded as the major class of fungal sphingolipids. A currently more acceptable name for this group is ceramides. Cerebrins have been

obtained from yeasts, filamentous fungi and mushrooms. Glycolipids based on glycerol have also been found. The principal components of glyceroglycolipids of plants are mono- and digalactosyldiacylglycerols. A large number of glycosyldiacylglycerols have also been isolated from bacterial species. Mono- di, tri- and tetraglycosyl derivatives have been found in which glucose, galactose, mannose, rhamnose or glucuronic acid, in various molar proportions, are the monosaccharide units. One or more fatty acid residues may also be esterified to the sugar components. The occurrence of this complex group of lipids, in fungi, has been reviewed by Weete (1980).

Yeasts secrete complex extracellular lipids known as sophorosides and ustilagic acids, which are hydroxy acid glycosides. Hydroxy acid glycosides are also reported to occur in moulds. Laine *et al.* (1972) described a major lipid from *Aspergillus niger* as a monoglucosyloxyoctadecenoic acid, which is apparently membrane associated, and is absent from the growth medium.

Glycosyl diglycerides, of the type prominent in Gram-positive bacteria and higher plants, have been reported to occur in yeasts and moulds, but no structural characterization of these lipids has been carried out (Brennan *et al.*, 1974).

Little is known about the function of fungal glycolipids. Some may be a major source of fatty acids and others appear to be endogenous reserves in spores, their metabolism seeming to depend on dormancy-breaking in fungal spores (Brennan *et al.*, 1974).

Aims of this work

The aims of the present investigation were:

(i) to determine the feasibility of employing a parasexual genetic program in order to obtain improved strains of *A. nidulans* for

lipid production

(ii) to optimise culture conditions for maximal lipid production from *A. nidulans*

(iii) to undertake qualitative and quantitative analysis of the lipid components of *A. nidulans* strains

(iv) to determine the influence of two extraction methods on the resulting lipid composition.

II. MATERIALS AND METHODS

1. Organisms

The wild types of *A. nidulans* used were from the Commonwealth Mycological Institute (CMI) (Kew, Surrey, England) and all auxotrophic mutants were derived from Glasgow stocks*. The origin and location of the mutants can be found in Barrat, Johnson and Ogata (1965) and Ball and Azevedo (1964).

The wild types were the following:

15. isolated from soil, CMI register no. 158,753

16. isolated from soil, CMI register no. 16,040

45. isolated from gum arabic, CMI register no. 45,625

The mutants differing in their nutritional requirements and conidial colours, were:

ane bio - thiamine and biotin requirement

bio met - biotin and methionine requirement

MSE - master strain E-markers in all eight linkage groups

ynic 5 ribo 5 - yellow conidia, niacin and riboflavin requirement

ypro 1 paba 6 - yellow conidia, proline and p-amino benzoic acid requirement

yw 3 s 12 nic 2 - white conidia, thiosulphite and niacin requirement

2. Media

2.1 Minimal medium (MM) (Pontecorvo *et al.* 1953)

$NaNO_3$	6.00 g
KCl	0.52 g
$MgSO_4.7H_2O$	0.52 g
KH_2PO_4	1.52 g
$ZnSO_4$	traces

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* (Department of Genetics, Glasgow University, Scotland.)

$FeSO_4.7H_2O$	traces
Glucose	10.00 g
Distilled water	1000 ml

pH adjusted to 6.5 with NaOH. When solid medium was desired 1.5 percent agar was added before autoclaving.

2.2 Complete medium (CM) (Pontecorvo *et al.* 1953)

Identical to MM except made up with tap water and supplemented with:

peptone	2.0 g
yeast extract	0.5 g
casein hydrolyzate	1.5 g
vitamin solution*	1 ml

* Vitamin solution

p-aminobenzoic acid	10.0 mg
pyridoxine	50.0 mg
aneurine	10.0 mg
niacin	100.0 mg
biotin	0.2 mg
riboflavin	100.0 mg
distilled water	100 ml

Kept in a dark flask at 4°C over chloroform after Tyndal sterilization.

2.3 Modified minimal medium (MMM)

Liquid minimal medium was modified for various fermentations in four ways:

a. glucose increased to 3% (MM + 3% glucose)

b. glucose increased to 17% (MM + 17% glucose)

c. glucose increased to 12%, KH_2PO_4 to 0.5% and $NaNO_3$ decreased to 0.1% (MM + 12% glucose + 0.5% KH_2PO_4 + 0.1% $NaNO_3$)

d. the same as medium c, but KH_2PO_4 concentration unchanged (MM + 12% glucose + 0.1% $NaNO_3$)

In all cases $MgSO_4.7H_2O$ was prepared separately as a stock solution and added to the medium after sterilization, to avoid precipitation of magnesium phosphate.

2.4 Medium of Naguib and Saddik (1960) (N&S) and Garrido, Gad and Walker (1958) (GGW) (g/100 ml)

	N&S	GGW
$NaH_2PO_4.2H_2O$	0.730	0.216
$MgSO_4.7H_2O$	0.022	0.072
K_2PO_4	0.500	0.066
$ZnSO_4.7H_2O$	0.005	0.013
$FeCl_3.6H_2O$	0.016	0.040
$NaNO_3$	0.607	0.300
glucose	17.000	9.000

pH 3.8 or 6.5

N&S medium was identical to that of Garrido and Walker (1956) with the exception of nitrogen source which was NH_4NO_3 (3 g/100 ml), in the latter.

3. Sterilization of media

The sterilization of media with increased amount of sugar, was by autoclaving at 10 lb/in^2 for 15 min to avoid sugar caramelization.

The sterilization of other media, solutions and fermenter vessel, was carried out at 15 lb/in^2 for 20 min.

4. Heterokaryon and diploid formation

The techniques employed for heterokaryon and heterozygous diploid formation were those described by Roper (1952) which are based on the ability of heterokaryon and diploid strains, but the inability of mutants, to grow in MM.

Liquid MM + 2 percent CM (CM was added to ensure a small initial growth) was inoculated with an equal amount of spore suspension from two mutants requiring different nutritional supplements. After two days incubation the mycelial mat was transferred to dishes of solid MM where, after 5 to 10 days, small patches of heterokaryotic mycelium may arise from point inocula. Heterokaryons thereafter were kept in MM.

Diploid colonies were isolated from the conidia of a balanced heterokaryon plated on MM. If heterozygous diploid nuclei have arisen by fusion between nuclei (one of each parental kind), the conidia carrying these will give rise to 2 N colonies, able to grow in non-supplemented medium.

Subsequent identification of diploid colonies was carried out on the basis of their conidial diameter (Pontecorvo *et al.* 1953, Clutterbuck, 1969).

5. Preparation of inocula

Conidia from colonies grown on solid medium for 3-5 days, at 37°C, were suspended in Tween 80 (0.1%, v/v) and agitated in a mixer to break down the conidial chains.

The conidia were then suspended in NaCl solution (0.89%, w/v) and the suspension diluted to give a concentration of 1×10^6 conidia/ml. 0.1 ml of this solution was used to inoculate 100 ml of suitable medium.

The estimation of conidial number was carried out using a haemocytometer.

6. Culture conditions

Cultures were grown as surface cultures (unagitated) in an incubator or in batches using an orbital shaker (LH Engineering) (rotation setting at mark 6) or a New Brunswick MF114 fermentor with an aeration rate of 0.6 litre air/min/litre of culture, and an agitation speed of 400 r.p.m.

A vortex-stirring apparatus, similar to that described by Harvey, Fewson and Holms (1968), was also used. This device consists of five flasks of 1 l capacity immersed in a controlled temperature water bath. The agitation for each flask is provided by a magnetic stirrer bar which is located over a rotating magnet. The speed of rotation (setting at 6) was adjusted to give maximum aeration.

The amount of medium, growth temperature and time of harvesting used in each culture method are detailed in Results.

7. Harvest and dry weight determination

The mycelial mats and pellets were harvested by vacuum filtration and washed twice with distilled water. The stirred cultures were harvested by centrifugation at 38,000 g and washed by resuspending the cell pellets in distilled water and centrifugation as above.

After washing, the cells were immediately frozen with liquid nitrogen. The frozen cells were then freeze-dried overnight and the dry weight determined.

8. Estimation of residual glucose

8.1 DNSA method (Bruner, 1964)

Reagent: 20 g of 3,5-dinitrosalicylic acid (DNSA) (BDH Chemicals Ltd, England) dissolved in 700 ml of 1 N sodium hydroxide, and diluted to 1000 ml with distilled water. This solution was filtered and stored in polyethylene bottles at room temperature.

Method: 1.0 ml of the sample to be analysed containing up to 14 micromoles of D-glucose was pipetted into 2 ml of chilled DNSA reagent and diluted to 4 ml with distilled water. The contents were vortex-mixed and placed on ice until thoroughly chilled. A marble was used to seal the tube which was placed in a boiling water bath for exactly 5 min. The resulting mixture was diluted with 8 ml of distilled water, vortex-mixed and the absorbance determined immediately at 540 nm. The method was calibrated with a concentration series ranging from 0.5 to 14 micromoles of glucose.

8.2 Glucose oxidase method (Updike and Hicks, 1967)

This method is based on the oxidation of glucose by glucose oxidase:

glucose + oxygen → gluconic acid + hydrogen peroxide

Oxygen uptake is monitored using the oxygen electrode.

Reagents: 1. phosphate-citrate buffer 25 mM, pH 5.0 (121.5 ml citric acid (0.1 M) + 128.5 ml dibasic sodium phosphate (0.2 M), pH adjusted to 5.0 and made up to 1 litre with distilled water.

2. glucose 0.1 percent (in 1, w/v)

3. glucose oxidase: 300 µg dissolved in 10 ml 1.

Method: The sample (containing 0.5 to 5.5 micromoles of glucose) was placed in the reaction vessel of the oxygen electrode (Yellow Springs) at 40°C. 50 µl of glucose oxidase reagent were added by microsyringe and the oxygen uptake recorded.

Glucose concentrations were calculated from a standard curve prepared with D-glucose.

9. Nitrate determinations

Reagent: Equal volumes of conc. phosphoric acid and conc. sulphuric acid were mixed and allowed to stand for one week. This minimises the possible nitrate contamination present in the acids.

Szechrome (Yedatek Ltd., Israel) was added to the acids (0.5 percent, w/v) with shaking until solution was achieved.

Method: 0.5 ml sample (containing up to 100 nmol nitrate) was pipetted into a test tube containing 5 ml reagent. The tube was sealed with parafilm and inverted several times to mix. The developed colour was read immediately at 570 nm and converted to nmol nitrate by reference to a standard curve prepared at the same time, ranging from 10 to 100 nmol of nitrate.

10. Lipid extraction methods

All solvents used for extraction and chromatography were redistilled and 50 mg per litre butylatedhydroxytoluene (BHT) (Sigma) was added as an antioxidant.

10.1 Extraction with chloroform-methanol (adapted from Kates, 1982)

This method was normally used to determine the total lipid of the samples, using 0.25 g of dried mycelium or 10 g in "large scale" experiments.

The freeze-dried mycelium was rehydrated by adding 0.1 M potassium chloride to give a final concentration of approximately 50 mg/ml and left 15 min at 4°C. The suspension was blended in a Waring blender for 3 min with chloroform-methanol (1:2, v/v) (3.75 ml per ml of suspension) and left at room temperature for 2 h, shaking occasionally. After centrifugation in 30 ml glass stoppered centrifuge tubes, the supernatant extract was decanted into another tube and the residue resuspended in methanol-chloroform-0.1 M potassium chloride (2:1:0.8, v/v) (4.75 ml per ml of original suspension) and re-extracted as above. To the combined supernatant extracts, were added chloroform + 0.1 M potassium chloride (2.5 ml of each, per ml of original suspension), and the mixture was centrifuged. The lower chloroform

phase was withdrawn carefully with a Pasteur pipette and transferred to a 50 ml tared r.b. Quickfit flask. The solvent was removed with a rotary evaporator and traces of water eliminated by the repeated addition and evaporation of absolute ethanol. The lipid residue was weighed, transferred to a small sample vial and stored at -15°C.

For large scale lipid extraction this procedure was scaled up proportionally and the extraction was carried out in an appropriately sized glass stoppered Erlenmyer flask. The extraction mixture was separated in separatory funnels.

10.2 Extraction with isopropanol (modified from Kates, 1982)

Isopropanol was used in "large scale" lipid extraction in order to compare it with the chloroform-methanol method. It is known that chloroform is a very useful solvent for removing non-polar lipids, but also along with several other organic solvents, promotes the action of phospholipase D, normally present in some plant tissues (Kates, 1982).

Phospholipase D is a very stable enzyme and causes enzymatic degradation of lipids during the extraction process. However, hot isopropanol can inhibit this enzyme (Kates, 1982).

To 10 g of freeze dried mycelium, 300 ml of hot isopropanol was added and the mixture was blended for 2 min in a Waring blender. The hot homogenate was filtered with suction and the residue was washed with hot isopropanol (200 ml). The filter cake was then blended with chloroform-methanol (1:1, v/v) (200 ml) and filtered. The residue was washed with chloroform-isopropanol (1:1, v/v) and finally with chloroform (200 ml, each). The combined filtrates were concentrated on a rotary evaporator, the residual lipids were taken up in chloroform (200 ml) and the solution was washed several times with 1 percent sodium chloride solution (100 ml portions). The solvent phase was evaporated, the lipid redissolved in ethanol and concentrated to dryness to remove any traces of water, transferred to a small sample vial and stored at -15°C.

11. Preparation of fatty acid methyl esters (FAME)

Determination of the fatty acid composition of lipids by gas chromatography is usually preceded by esterification or transesterification to form the methyl esters which increases the volatility of these compounds and makes them more suitable for analysis. Acid-catalysed esterification and transesterification (Christie, 1982) was used.

The lipid sample (up to 50 mg) was dissolved in benzene (1 ml) in a r.b. Quickfit flask (25 ml) and a solution of methanol-conc. sulphuric acid (80:2, v/v) (2 ml), was added. The mixture was refluxed for 2 h, then sodium chloride (5 percent, v/w) (5 ml) was added and the required esters were extracted with hexane (2 x 5 ml).

The hexane layer was washed with a solution of potassium bicarbonate (2 percent w/v) (4 ml) and dried over anhydrous sodium sulphate. The solution was filtered, the solvent removed under reduced pressure in a rotary evaporator and the lipid residue transferred to a small sample vial which was stored at $-15^{o}C$.

12. Gas liquid chromatography analysis of FAME

A Pye Unicam series 104 gas liquid chromatograph (Cambridge, England) equipped with a flame ionisation detector, was used for analysis of samples.

A glass column (2 m x 4mm) packed with 10% SP-2330 (Supelco, Inc. Bellefonte, PA, USA), on 100-120 mesh chromosorb WAW was used isothermally at $198^{o}C$ with a N_2 flow rate of 20 ml/min.

The sample (1 µl) was injected with a microsyringe (Terumo, Japan). The methyl ester peaks were identified by using authentic standards and tables of equivalent chain lengths (ECL'S) obtained from James plots of carbon number versus logarithm of retention time for saturated straight chain standards.

The area of each peak obtained was determined from the product of the peak height and its retention time (Carrol, 1961).

13. Lipid phosphorus determination

Phosphorus was determined in lipid fractions by the method of Rouser *et al.* (1966). All glassware was ore-soaked in non-phosphate containing detergent (5% Decon 90) (Decon Laboratories, Brighton, England) to remove any contamination by phosphorus, before use.

A small amount of lipid sample (or a spot scraped from TLC plates) was transferred to a 30 ml Kjeldahl tube, the solvent evaporated and 0.9 ml percloric acid (72 percent), was added. The mixture was digested under gentle reflux for twenty minutes in an electrically heated Kjeldahl rack within a fume cupboard. Vigorous boiling was avoided to prevent boiling off of any volatile components. The tubes were allowed to cool for 15 min and their insides rinsed down with distilled water (5 ml). Then, 2.5 percent (w/v) ammonium molybdate (1 ml), was added with swirling to mix the contents, followed by freshly prepared 10 percent (w/v), ascorbic acid (1 ml) and distilled water (2 ml). This entire mixture was transferred to a 15 ml test tube. A marble was put over the mouth and the tube was heated on a boiling water bath for 5 min. After cooling it was centrifuged (in the case of material from TLC) in a bench centrifuge to precipitate the silica. The optical density of the solution was then determined at 820 nm.

A calibration curve was plotted using Na_2PO_4 solution (stock: 5 µg phosphorus per ml). The standard solutions were not digested. The calibration graph was linear up to 10 µg phosphorus.

To correct P to phospholipid content the multiplication factor was 25, since the proportion of phosphorus is 5 percent (w/v), in most commonly occurring phospholipids. with the exception of cardiolipin and lysophosphatides.

14. Thin layer chromatographic analysis

14.1 Preparation of analytical and preparative plates

Silica gel G was used for separation of neutral lipids and silica gel H for polar lipids.

The adsorbent, for preparative plates, was pre-washed with chloroform-methanol (1:1, v/v) and the thickness of the adsorbent layer was 0.5 mm. Silica gel G (about 45 g) was mixed with water (80 ml) to form a slurry which was spread onto 5 glass plates (20 cm^2) with an applicator.

For analytical plates, the thickness of the adsorbent layer was 0.25 mm. Silica gel G plates were prepared using 30 g of adsorbent and 60 ml of water and, silica gel H plates, with 25 g of adsorbent mixed with 55 ml of water.

The adsorbent was allowed to solidify for a while, the plates stored out of contact with contaminating vapour and reactivated at 120°C for 1-2 h before use.

14.2 Application of sample

The lipid mixture was applied, as soon as the activated plate had cooled, with a graduated microsyringe (Terumo, Japan) as spots (about 10 µg of each individual lipid), or as streaks 1.5 cm apart and 1.5 cm from bottom of plate. The plate was chromatographed immediately.

14.3 Development

The plates were developed in tanks lined with chromatographic paper (Whatman, 3 MM) to aid in saturating the tank with solvent vapour. One or two plates were placed in the tank and the solvent was allowed to ascend to within about 1-2 cm of the top of the plate. The plates were dried using a hair drier for 15 min and after the solvent had been completely removed, the plates were stained.

14.4 Choice of solvents for 1-D TLC

14.4.1 Neutral lipid separation

The optimum solvent system was determined by testing some of the usual solvents used to separate non-polar lipids.

The following solvents were tested:

a. hexane-diethyl ether-formic acid (80:20:2, v/v) (Storry and Tuckley, 1967)

b. benzene-diethyl ether-ethyl acetate-acetic acid (80:10:10:0.2, v/v) (Storry and Tuckley, 1967)

c. hexane-diethyl ether-acetic acid (80:20:1, v/v) (Mangold, 1969)

14.4.2 Polar lipid separation

The polar lipids were separated using chloroform-methanol- di iso butyl ketone-acetic acid-water (45:15:30:20:4, v/v) (Hunter *et al.*, 1981) run at 4°C.

14.5 2-D TLC of polar lipids

Two dimensional TLC was carried out on commercial plates (20 cm^2) pre-coated with 0.25 mm layers of silica gel 60 H (Merck, Germany). The plate was activated at 120°C for 1 h, before use, allowed to cool and the sample applied on top of a small spot of rhodamine solution (0.0012 percent in chloroform-methanol 1:1, v/v), in the lower right-hand corner of the plate. Rhodamine was used to help to locate both the origin and solvent front and to avoid the possibility of running the plate twice in the same direction.

14.5.1 Development of plates

Tanks containing solvent were lined with chromatography paper (Whatman 3 MM) sealed by a strip of 2 inch wide parcel tape around the rim and allowed to saturate at 4°C for at least 1 h before use. Low temperature

was felt to be desirable since it improves resolution by minimising diffusion of spots and autoxidation of polyunsaturated fatty acids.

Chromatography, in the first dimension was carried out to within 0.5 cm of the top (120 min), the plate removed from the tank, dried using a hair drier for 15 min, turned through 90 degrees and developed in the second dimension for the same distance (180 min).

The plate was then removed and dried as above.

14.5.2 Selection of solvent systems

Solvent mixtures were made up daily to avoid possible chemical interactions between components and losses by evaporation.

The following solvent systems were tried.

a. Hunter *et al.*, 1983:

first dimension: chloroform-methanol-ammonia (25%, w/v) - water (160:88:11:11, v/v)

second dimension: chloroform-methanol-acetic acid (50:27:12, v/v)

b. Modified from Hunter *et al.*, 1983

first dimension: chloroform-methanol-ammonia (25%, w/v) - water (160:88:11:11, v/v)

second dimension: chloroform-methanol-di iso butyl ketone-acetic acid-water (45:15:30:20:4, v/v)

c. Modified from Hunter *et al.*, 1983; Hunter *et al.*, 1981

first dimension: chloroform-methanol-acetic acid (50:27:12, v/v)

second dimension: chloroform-methanol-di iso butyl ketone-acetic acid-water (45:15:30:20:4, v/v)

14.6 Detection of lipid components

After all solvent had been completely removed by drying the plates under a warm hair drier, the following stains were used for detection and identification of lipids:

a. Iodine (Mangold and Malins, 1960)

The plate was inserted into an all glass chromatographic tank containing 5-10 g of iodine crystals. The tank was gently warmed by a hair drier to hasten sublimation of I_2.

This reagent was used as a general detection reagent, since both saturated and unsaturated lipids appear as brown-yellowish spots on a pale-yellow background.

b. Rhodamine 6G (Marinetti, 1964)

The plate was sprayed with rhodamine (0.005 percent, w/v) and examined wet under ultraviolet light (366 nm). Basic and neutral lipids appear as pink or yellow spots; acidic lipids as blue or purple spots.

c. Anthrone (Galliard, 1968)

The plate was sprayed with anthrone solution (0.2 percent w/v, anthrone : conc-sulphuric acid) and heated on a hot plate at 70°C for 20 min.

Glycolipids appear as blue-green or pink spots on a white background; phospholipids and other lipids stain grey-brown.

d. Ninhydrin (Marinetti, 1964)

Reagent grade ninhydrin (0.25 g) was dissolved in 100 ml of acetone-lutidine (9:1, v/v), freshly prepared, and sprayed. The phospholipids with NH_2 groups (phosphatidylethanolamine, phosphatidylserine and their derivatives) appear as purple spots. The chromatogram may be subsequently stained with rhodamine, in order to

locate other lipid components.

e. Antimony molybdate (Eastman Organic Chemicals Bulletin, 1976)

Reagents: 1. Sulphuric acid 2.5 M : 70 ml of H_2SO_4 in 500 ml of water

2. Antimony potassium tartrate solution : 1.37 g of $K(ScO)\ C_4H_4O_6.\frac{1}{2}H_2O$ in 500 ml of water

3. Ammonium molybdate solution : 20 g of $(NH_4)_6\ Mo_7\ O_{24}.4H_2O$ in 500 ml of water

4. Ascorbic acid solution : 1.86 g of ascorbic acid in 100 ml of water.

All above solutions are stable for at least 8 weeks in the refrigerator, with the exception of ascorbic acid, which is stable for 1 week only. A "combined reagent" must be prepared with all above reagents at room temperature using 25 ml of 1 + 2.5 ml of 2 + 7.5 ml of 3 + 15 ml of 4.

The detection reagent was prepared mixing the following:

"combined reagent"	8 ml
isopropyl alcohol	1 ml
distilled water	13 ml

The plate was sprayed with the freshly prepared detection reagent and heated for 1-2 min at 70°C on a hot plate. The phospholipids sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidic acid (PA) appear as blue spots and phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) as grey spots.

f. Ferric chloride (Lowry, 1968)

Reagents: Ferric chloride reagent was prepared by dissolving 50 mg of $FeCl_3.6H_2O$ in 90 ml of water and 5 ml of glacial acetic acid. 5 ml of conc. sulphuric acid was then added. This reagent is stable for 3

months, at room temperature.

Method: The plate was sprayed with the reagent and heated at 100°C for 1-2 min. Sterol and sterol-ester appear as violet spots, and other lipids as brown, on a white background.

g. Sulphuric acid-acetic acid (Jatzkewitz and Mehl, 1960)

Reagent: Sulphuric acid-glacial acetic acid (1:1, v/v). After spraying the plate, it was heated at 90°C for 15 min. Sterol and sterol-ester appear as red spots on a white background.

15. Lipid class separation by Florisil column chromatography (Kates, 1982)

The major lipid classes and the sub-classes of neutral lipids present in the mixtures obtained from isopropanol and from chloroform-methanol, large-scale extractions, were separated on acid treated Florisil columns. Preliminary separation by elution of the acid treated Florisil column was carried out with the solvent sequence:

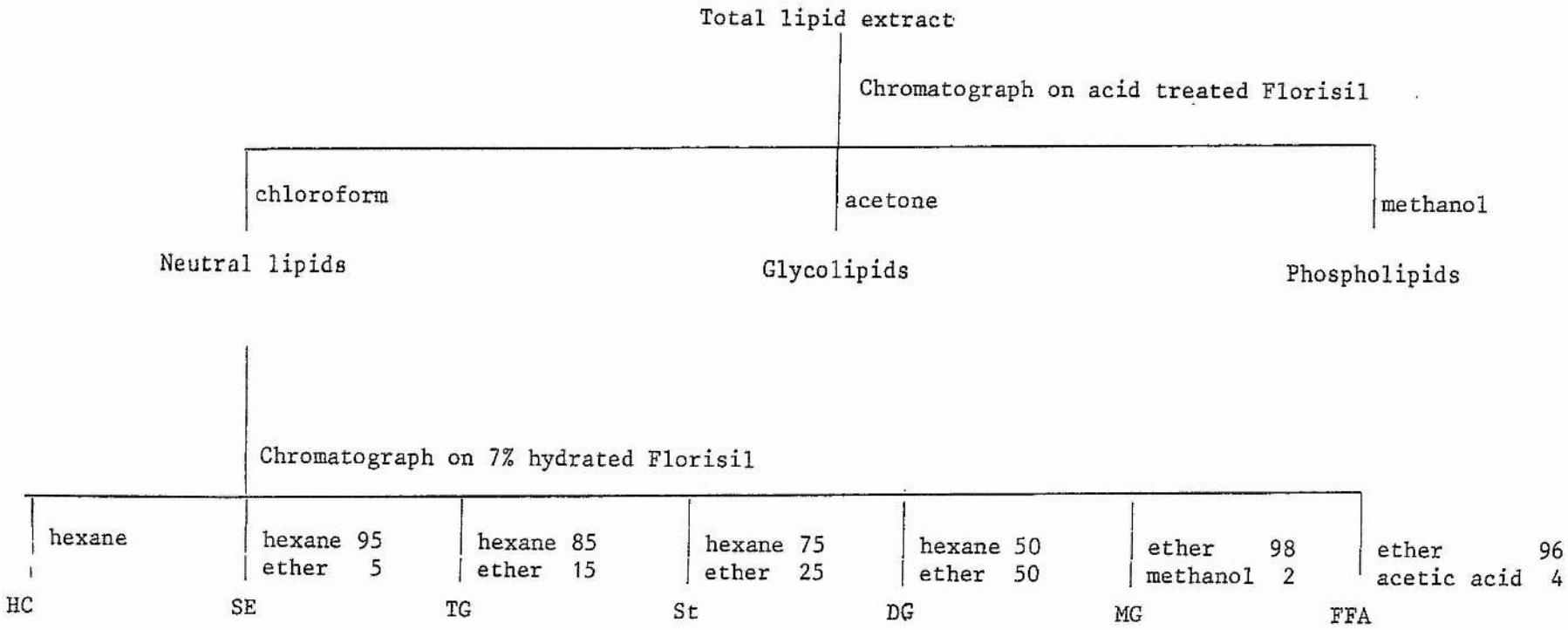
chloroform → acetone → methanol, which eluted respectively:
neutral lipids, glycolipids and phospholipids.

The neutral lipids were further fractionated on a column of acid treated Florisil (7% hydrated).

A flow diagram is shown (Fig. 1) which summarizes these methods.

15.1 Preparation of acid treated Florisil (Carrol, 1963, Carrol *et al.*, 1968)

Concentrated hydrochloric acid (900 ml) was mixed with 300 g Florisil (80-100 mesh, BDH Chemical Ltd, England) and heated on a steam bath for 3 h. The hot supernatant was decanted and the adsorbent was washed with acid (about 150 ml) and heated overnight with a further 900 ml of acid. The supernatant was decanted and the residue was



Abbreviations: HC = hydrocarbons, SE = steryl esters, TG = triglycerides, St = sterols, DG = diglycerides, MG = monoglycerides, FFA = free fatty acids

FIGURE 1. Scheme (from Marinetti, 1969) for isolation and separation of lipid classes and neutral lipid sub-classes by column chromatography.

filtered in a Buchner funnel and washed with distilled water till neutral. The residue was sucked dry and transferred to a glass dish and heated at 120°C overnight. The acid treatment and water washing steps were repeated, as above. The adsorbent was washed on the Buchner funnel with 400 ml each of methanol, methanol-chloroform (1:1, v/v), chloroform, and finally ether (all freshly distilled) and then activated by heating at 120°C, overnight, before use.

To prepare 7 percent hydrated Florisil, water (7 ml) was added to acid treated Florisil (100 g) in a stoppered flask and shaken overnight on a mechanical shaker.

15.2 Packing of the column

A glass column (2 x 40 cm) with a sintered disc at the bottom to support the adsorbent with a small amount of Hyflo on the top of the sintered disc, was used. The Hyflo was used to minimize blocking of the sinter by the adsorbent.

The adsorbent (48 g) was packed as a slurry in the first solvent to be used in the separation and poured into the chromatography tube.

15.3 Application of sample and elution of column

To load the column the overlying solvent level was allowed to fall to the top of the column bed and the sample (dissolved in the first elution solvent) applied carefully down the side of the column to minimize disturbance of the adsorbent. The sample was allowed to percolate into the adsorbent, flow being stopped when the level again reached the top of the bed. The solvent reservoir was then attached and the first eluting solvent carefully allowed to flow into the column.

Elution was carried out at a flow rate of about 3 ml/min.

Chromatography on the acid washed Florisil column was usually carried out with a loading of 400 mg of total lipid dissolved in 20 ml of chloroform, each time. The volume of each eluting solvent was c.a.

4.5 column volumes (270 ml).

Column chromatography of neutral lipids on 7 percent hydrated acid washed Florisil was also carried out using a loading of 400 mg neutral lipid, but dissolved in 10 ml of hexane. The elution sequence (Kates, 1982) was as follows:

Eluting solvent	Amount (ml)
hexane	160
hexane-ethyl ether (95:5)	362
hexane ethyl ether (85:15)	600
hexane-ethyl ether (75:25)	400
hexane-ethyl ether (50:50)	400
ethyl ether-methanol (98:2)	540
ethyl ether-acetic acid (96:4)	200

15.4 Detection and quantitation of eluted lipids

Quantitation of each lipid class eluted in the different column fractions was performed gravimetrically after fine filtration to remove Florisil particles. Ethanol was added during rotary evaporation of the solvents to assist in removal of residual traces of water.

The lipid residues were transferred to small sample vials dried in a stream of N_2 and stored at $-15^{o}C$. The purity of each fraction was checked by TLC.

Phosphorus estimation was also carried out as a check for efficiency of separation on the neutral, glyco- and phospholipid fractions as described in 13.

16. Fractionation of neutral lipid by preparative TLC (Kates, 1982)

Narrow streaks of the total lipid (1 mg/cm) along with standards were applied to preparative TLC plates, and these were developed in hexane-ether-acetic acid (80:20:1, v/v).

The dried plates were sprayed with rhodamine 6G (0.005 percent w/v) and viewed while wet under ultraviolet light. The bands observed were marked, identified and each scraped off into a glass stoppered tube containing ether (2 ml). This was shaken and the ether transferred to another tube. The silica was then re-extracted as above. The combined eluant and washings were fine filtered, transferred to a round-bottomed flask, diluted with an equal volume of benzene and evaporated on a rotary evaporator under reduced pressure at 30°C.

The residue was stored in a small sample vial at -15°C for further analysis.

III. RESULTS

1. Isolation of heterokaryons and heterozygous diploids of *A. nidulans*.

Fifteen heterokaryons and fifteen heterozygous diploids were isolated from six paired combinations of auxotrophic mutants of *A. nidulans*. The spore diameters of the mutants and diploids are shown in Table 1. The results represent the mean of fifty conidia of each strain. The diploid strains, characteristically exhibited larger spore diameters than their respective complementary biochemically deficient parents.

TABLE 1. Spore diameter (mean of 50 conidia) of haploid and diploid strains of *A. nidulans*.

strain	ploidy	spore diamenter (µm)
bio met	N	0.3246 ± 0.031
ane bio	N	0.3258 ± 0.028
ynic 5 ribo 5	N	0.3200 ± 0.025
ypro 1 paba 6	N	0.3466 ± 0.031
yw 3 s 12 nic 2	N	0.3208 ± 0.031
MSE	N	0.3166 ± 0.029
ane bio // bio met	2N	0.4269 ± 0.033
bio met // ynic 5 ribo 5	2N	0.4184 ± 0.032
bio met // ypro 1 paba 6	2N	0.4670 ± 0.034
bio met // yw 3 s 12 nic 2	2N	0.4314 ± 0.039
bio met // MSE	2N	0.4286 ± 0.036
ane bio // ynic 5 ribo 5	2N	0.3708 ± 0.010
ane bio // ypro 1 paba 6	2N	0.4416 ± 0.043
ane bio // yw 3 s 12 nic 2	2N	0.4120 ± 0.034
ane bio // MSE	2N	0.4550 ± 0.037
ynic 5 ribo 5 // ypro 1 paba 6	2N	0.4716 ± 0.038
ynic 5 ribo 5 // yw 3 s 12 nic 2	2N	0.4544 ± 0.039
ynic 5 ribo 5 // MSE	2N	0.3818 ± 0.031
ypro 1 paba 6 // yw 3 s 12 nic 2	2N	0.4727 ± 0.037
ypro 1 paba 6 // MSE	2N	0.4334 ± 0.031
yw 3 s 12 nic 2 // MSE	2N	0.4018 ± 0.027

2. Assessment of lipid production by strains of *A. nidulans*.

2.1 Initial screening of strains

Firstly, two of the six auxotrophic mutants and one diploid were tested for their lipid producing ability in a medium with a simple composition: minimal medium with slightly more glucose (3 percent) than the optimum for growth (1 percent).

This choice of medium was because it promotes a fast growth rate and avoids dediploidization of the diploid strain (Ball and Azevedo, 1976).

Growth curves were determined in MM + 3% glucose (thiamine, biotin and methionine, 10 mg/ml medium, added to the growth medium of deficient mutants) in an orbital shaker at 37°C, using flasks of 250 ml capacity containing 100 ml of medium with the mutants: *bio met*, *ane bio* and the diploid *bio met // MSE*. The mutant *ane bio* was also grown in an incubator at 37°C, without agitation to determine the influence of aeration. Samples were taken every two days, harvesting three flasks each time. Lipid extraction was performed on the washed, dried and weighed mycelium. Sugar utilized was determined on the cell-free culture medium.

The results of these experiments are shown in Tables 2, 3, 4 and 5, and Figure 2.

Days of incubation	Biomass (g dry wt/100 ml)	Lipid (% dry wt)	Total lipid (mg/100 ml)
2	0.27 ± 0.02	3.98 ± 0.35	10.75
4	0.66 ± 0.04	3.78 ± 0.43	23.10
6	0.94 ± 0.12	4.06 ± 0.23	38.16
8	0.92 ± 0.12	3.98 ± 0.67	36.62
10	0.91 ± 0.00	4.97 ± 0.64	44.96
12	0.79 ± 0.12	5.41 ± 0.31	42.74

TABLE 2. Biomass and lipid content of mutant *bio met* grown in shaker at 37°C in MM + 3% glucose (biotin and methionine added). Each value is the mean of three replicates ± standard deviation.

Days of incubation	Biomass (g dry wt/100 ml)	Lipid (% dry wt)	% sugar consumed	Total lipid (mg/100 ml)
2	0.46 ± 0.05	3.81 ± 0.08	26.74	17.53
4	0.79 ± 0.22	3.39 ± 0.06	49.51	26.78
6	1.13 ± 0.03	4.61 ± 0.27	98.65	52.09
8	0.92 ± 0.17	4.99 ± 0.70	95.21	45.91
10	0.70 ± 0.01	4.65 ± 0.48	99.34	32.55
12	0.57 ± 0.07	5.07 ± 0.21	99.24	28.90

TABLE 3. Biomass, lipid content and sugar consumption of diploid *bio met // MSE* grown in shaker at 37°C in MM + 3% glucose. Each value is the mean of three replicates ± standard deviation.

Days of incubation	Biomass (g dry wt/100 ml)	Lipid (% dry wt)	% sugar consumed	Total lipid (mg/100 ml)
2	0.42 ± 0.03	4.91 ± 0.05	20.26	20.62
4	0.64 ± 0.11	4.56 ± 0.36	59.93	29.18
6	0.75 ± 0.17	5.81 ± 0.77	87.45	43.58
8	0.81 ± 0.13	5.87 ± 0.90	94.05	47.55
10	0.77 ± 0.09	5.57 ± 0.83	95.23	42.89
12	0.53 ± 0.28	6.10 ± 0.54	98.08	32.33

TABLE 4. Biomass lipid content and sugar consumption of mutant *ane bio* grown in shaker at 37°C in MM + 3% glucose (thiamine and biotin added). Each value is the mean of three replicates ± standard deviation.

Days of incubation	Biomass (g dry wt/100 ml)	Lipid (% dry wt)	% sugar consumed	Total lipid (mg/100 ml)
2	0.05 ± 0.00	5.51 ± -	2.08	2.70
4	0.19 ± 0.02	2.79 ± 0.32	8.33	5.30
6	0.35 ± 0.02	4.04 ± 0.12	21.25	14.14
8	0.46 ± 0.00	4.13 ± 0.25	34.55	19.00
10	0.73 ± 0.03	4.51 ± 0.25	68.44	32.92
12	0.88 ± 0.07	4.24 ± 0.11	70.90	37.31

TABLE 5. Biomass, lipid content and sugar consumption of mutant *ane bio* grown in incubator without agitation at 37°C in MM + 3% glucose (thiamine and biotin added). Each value is the mean of three replicates ± standard deviation.

Growth of these strains in the shaker showed that after 6-8 days all the glucose had been consumed and, as expected, the biomass and the percentage of lipid in the mycelium did not increase thereafter, so that no increase in the total lipid was observed after exhaustion of the carbon source.

The rate of growth in the incubator (Table 5) was slower than in the shaker (Table 4) and was found to be in the form of a felt, whereas in the shaker spherical pellets were formed. The lipid yield and sugar consumed in the incubator are lower than in the shaker so that subsequent experiments were carried out in the shaker.

2.2 Comparison of wild type, mutants and diploids in shaker culture: biomass and lipid yield.

As the above results did not show great differences in lipid content between the strains, a preliminary trial was carried out, under the same conditions as above, with three wild types, four haploid mutants and five diploids.

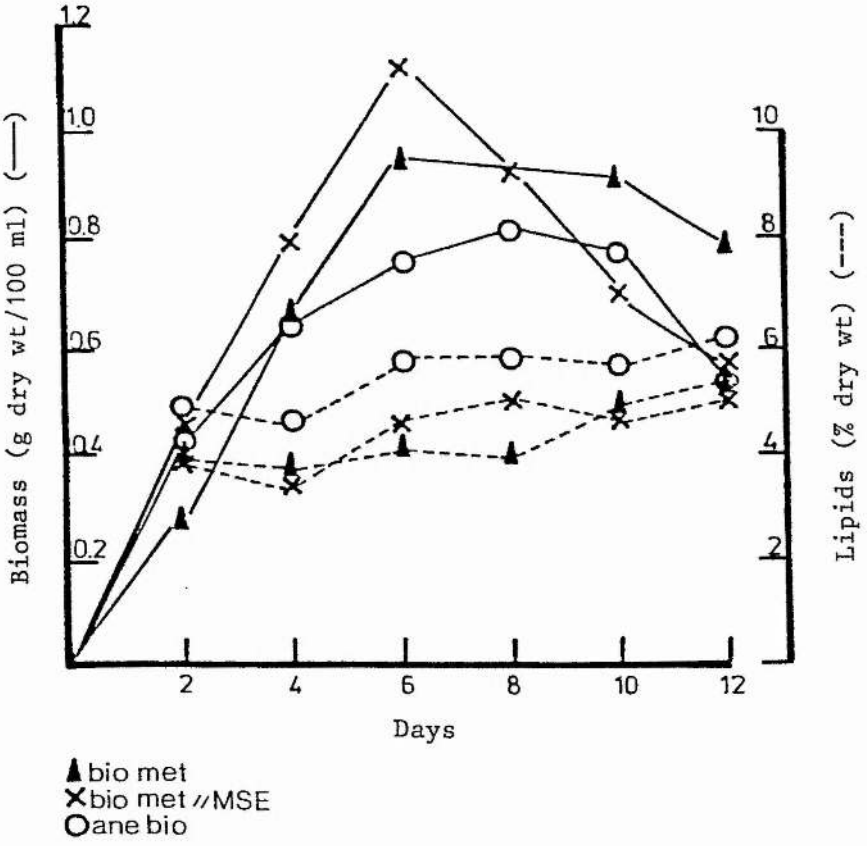


FIGURE 2. Biomass and lipid content of mutants and diploids of *A. nidulans* grown in MM + 3% glucose in orbital shaker.

Triplicate flasks of each strain were again grown for 8 days in 100 ml of medium, after which time the sugar consumption and lipid content were measured.

The results of these experiments are given in Table 6 and Figure 3. After 8 days, for all strains, almost all the glucose had been consumed and the lipid content was around 4-5 percent of dry weight. No strain demonstrated striking lipid accumulation.

2.3 Influence of medium composition and culture conditions on growth and lipid yield of wild types and diploids

Since others (e.g. Garrido *et al.*, 1958 and Naguib and Saddik, 1960) had obtained a high production of fat from *A. nidulans*, wild type, using a different medium from MM, it was decided to grow some strains under the same conditions defined by them.

Firstly, four diploids were grown as surface cultures under the same conditions used by Naguib and Saddik (1960) when the fat content (on a dry weight basis) was reported as 17.2 percent.

The 150 ml conical flasks, each containing 25 ml of medium, were incubated at 25°C over a period of 11 days. Under these conditions the maximum lipid content was found to be 3.84 percent, obtained from diploid *MSE // ane bio* which is even lower than that found in MM + 3% glucose.

The results of this experiment are shown in Table 7 and Figure 4.

Another experiment was carried out with the medium of Garrido *et al.*, (1958). This medium (100 ml) was used to grow wild type strains *15*, *16* and *45* at 37°C in an orbital shaker and, as in their experiments, growth was allowed to continue for 8 days. The results shown in Table 8 are each the mean of three replicates.

Although no increase in lipid content was found, the biomass was increased 2.5 times.

Strain	Biomass (g dry wt/100 ml)	Lipids (% dry wt)	% sugar consumed	Total lipid (mg/100 ml)
15	1.12 ± 0.01	4.71 ± 0.48	99.50	52.75
16	0.98 ± 0.01	4.76 ± 0.18	99.47	46.65
45	0.97 ± 0.05	4.45 ± 0.02	99.55	43.17
bio met	0.92 ± 0.12	3.98 ± 0.67	94.04	36.62
ane bio	0.81 ± 0.13	5.87 ± 0.90	87.07	47.55
MSE	1.14 ± 0.17	4.73 ± 0.11	99.10	53.92
ynic 5 ribo 5	0.71 ± 0.08	4.49 ± 0.34	99.07	31.88
ane bio // yw 3 s 12 nic 2	0.76 ± 0.22	4.93 ± 0.15	98.98	37.47
bio met // yw 3 s 12 nic 2	0.86 ± 0.17	4.88 ± 0.40	98.87	41.97
MSE // ane bio	1.03 ± 0.05	4.79 ± 0.20	99.20	49.34
bio met // ane bio	0.85 ± 0.14	4.75 ± 0.02	99.19	40.38
bio met // MSE	0.92 ± 0.17	4.99 ± 0.70	95.21	45.91

TABLE 6. Biomass, lipid content and sugar consumption of wild type, mutant and diploid strains of *A. nidulans* grown in 100 ml of MM + 3% glucose, in shaker at 37°C, for 8 days. Each value is the mean of three replicates ± standard deviation.

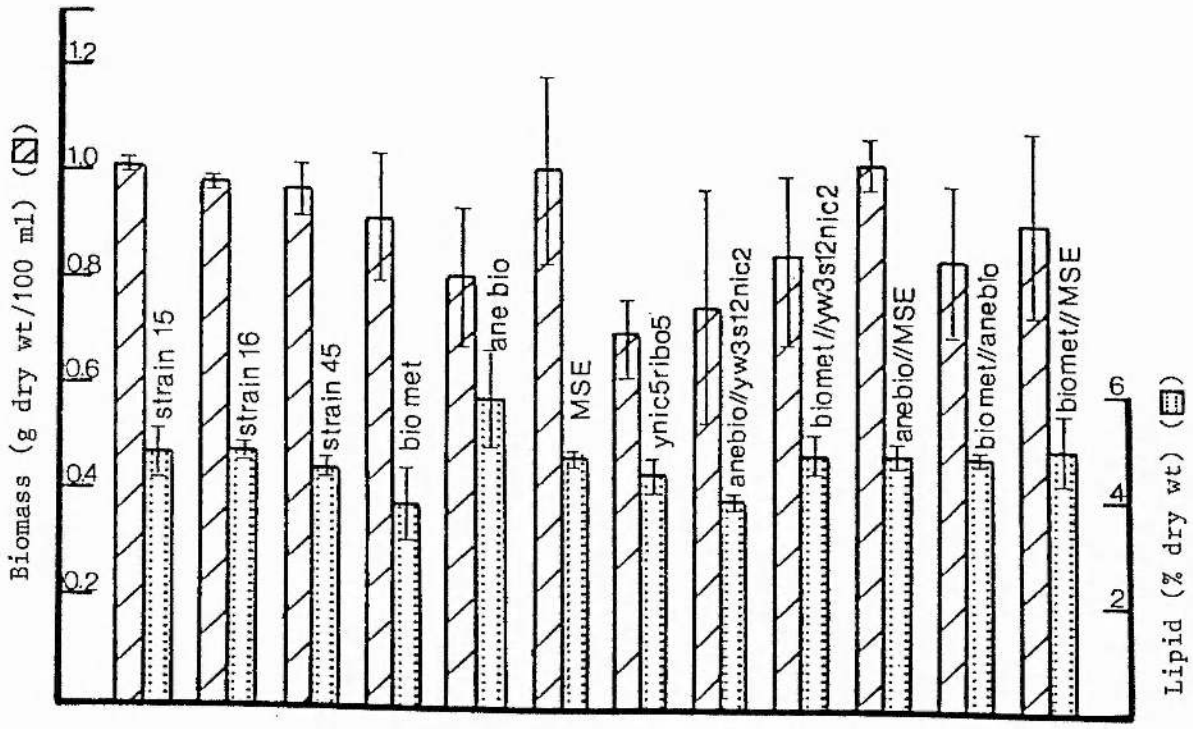


FIGURE 3. Biomass and lipid content of *A. nidulans* wild types, mutants and diploids grown in MM + 3% glucose in orbital shaker at 37°C.

Strain	Biomass (g dry wt/100 ml)	Lipid (% dry wt)	% sugar consumed	Total lipid (mg/100 ml
bio met // yw 3 s 12 nic 2	0.80 ± 0.12	2.76 ± 0.44	19.18	22.08
ane bio // yw 3 s 12 nic 2	0.74 ± 0.06	3.30 ± 0.10	23.43	24.42
ane bio // bio met	1.14 ± 0.32	2.62 ± 0.02	28.82	29.87
ane bio // MSE	1.31 ± 0.20	3.84 ± 0.16	33.64	50.30

TABLE 7. Biomass, lipid content and sugar consumption of diploid strains grown in 25 ml of N&S medium, pH 3.8, without agitation, at 25°C for 11 days. Each value is mean of three replicates ± standard deviation.

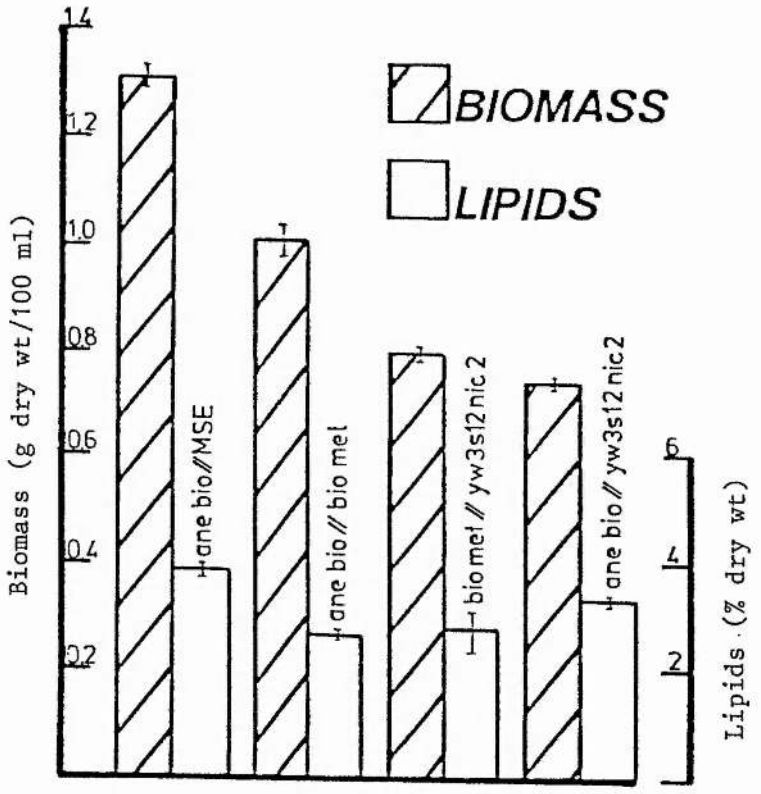


FIGURE 4. Biomass and lipid content of diploid strains of *A. nidulans* grown in N&S medium in incubator without agitation, at 25°C for 11 days.

Strain	Biomass (g dry wt/100 ml)	Lipid (% dry wt)	% sugar consumed	Total lipid (mg/100 ml)
15	2.24 ± 0.07	4.73 ± 0.10	71.59	105.95
16	2.52 ± 0.01	3.79 ± 0.08	62.22	95.51
45	1.76 ± 0.06	3.73 ± 0.29	78.15	65.65

TABLE 8. Biomass, lipid content and sugar consumption of three wild type strains, grown in GGW medium, in orbital shaker at 37°C, for 8 days. Each value is mean of three replicates ± standard deviation.

2.4 Influence of medium composition and culture conditions on growth and lipid production of wild type strain *16*.

It was clear from these results that the crosses carried out in this work did not produce strains whose lipid production was augmented, at least under the growth conditions used. Since the literature (see Introduction) suggested that the amount of lipid produced by at least one wild type strain of *A. nidulans* should have been considerably in excess of the values obtained here, further experiments were carried out with a single wild type, strain *16*, to investigate the influence of culture conditions on lipid production.

2.4.1 Effect of gradual increase of sugar in the medium

As high initial concentrations of sugar in the medium might inhibit growth by affecting the metabolism of the mould, a gradual increase in glucose was achieved by adding a Tyndallised solution of glucose every two days of incubation. Strain *16* was grown in 100 ml of MM + 3% glucose in an incubator without agitation, at 37^{o}C and glucose solution was added after 2, 4 and 6 days of cultivation. Each addition increased the total concentration of glucose by 2 percent. The results are shown in Table 9.

The biomass yield was improved, but the percentage of lipid in the dried mycelium was not affected.

Days of incubation	Total sugar added to the medium (%)	Biomass (g dry wt/100 ml)	Lipids (% dry wt)	% sugar consumed	Total lipids (mg/100 ml)
8	7*	0.75 ± 0.05	4.70 ± 0.02	57.19	35.25
8	9	1.25 ± 0.25	4.82 ± 0.26	39.93	60.25
10	9	1.38 ± 0.15	4.52 ± 0.20	54.77	62.38
13	9	1.73 ± 0.04	4.68 ± 0.24	73.68	80.96

* This series of flasks received additions of sugar at 2 and 4 days only.

TABLE 9. Biomass, lipid content and sugar consumption of strain *16*, grown in 100 ml of MM + 3% glucose in an incubator at 37°C, adding 2 g/100 ml extra glucose after 2, 4 and 6 days of incubation. The results are mean of three replicates ± standard deviation.

2.4.2 Fermenter growth - Naguib and Saddik (1960) medium

To test the effect of greatly increasing the total C and improving gas transfer, strain *16* was grown in a new Brunswick Fermenter in N&S medium (17% glucose), at 30°C over a 12 day period.

A spore suspension (5 ml) of 1 x 10^6 conidia/ml was used to inoculate 5 l of medium, directly into the fermenter vessel. Samples (100 ml) were harvested every day for one week and then at 9th and 12th days. The mycelial mass grew in the form of spherical pellets. At the end of the incubation period, two-thirds of the total dry mycelium came from the fermenter wall, where it had collected, above the level of the medium.

The results of this experiment are shown in Table 10 and Figure 5. After four days incubation, the lipid content peaked and remained constant around six percent. The nitrogen source was totally consumed after 6-7 days and there was still unutilized sugar at the end of the incubation period. Although the nitrogen had expired, the dry weight and therefore the total lipid yield, were still increasing. The pH decreased gradually over the incubation period.

Days of incubation	Biomass (g dry wt/100 ml)	Lipid (% dry wt)	% sugar consumed	% $NaNO_3$ consumed	pH	Total lipid (mg/100 ml)
1	0.13	-	2.80	40.87	5.9	-
2	0.39	2.72	5.74	31.66	6.0	10.61
3	0.80	4.40	7.08	54.66	5.9	35.20
4	0.77	6.04	13.63	66.69	5.3	46.51
5	1.19	6.00	24.71	94.48	5.3	71.40
6	1.31	6.04	28.74	98.83	5.3	79.12
7	1.63	6.12	37.44	100	4.9	99.76
9	1.83	6.08	47.91	100	4.8	111.26
12	0.98*	6.68	63.08	100	4.8	174.35

* This figure is the mycelium weight remaining in the medium. However, much wall growth was also apparent at this final harvest, amounting to 67.8 g. Total biomass yield (wall grown mycelium + mycelium from the medium) for 5 l = 116.8 g ≡ 2.34 g/100 ml.

TABLE 10. Biomass, lipid content, nitrogen and sugar consumption of wild type *16*, grown in 5 l of N&S medium in a new Brunswick Fermenter at 30°C.

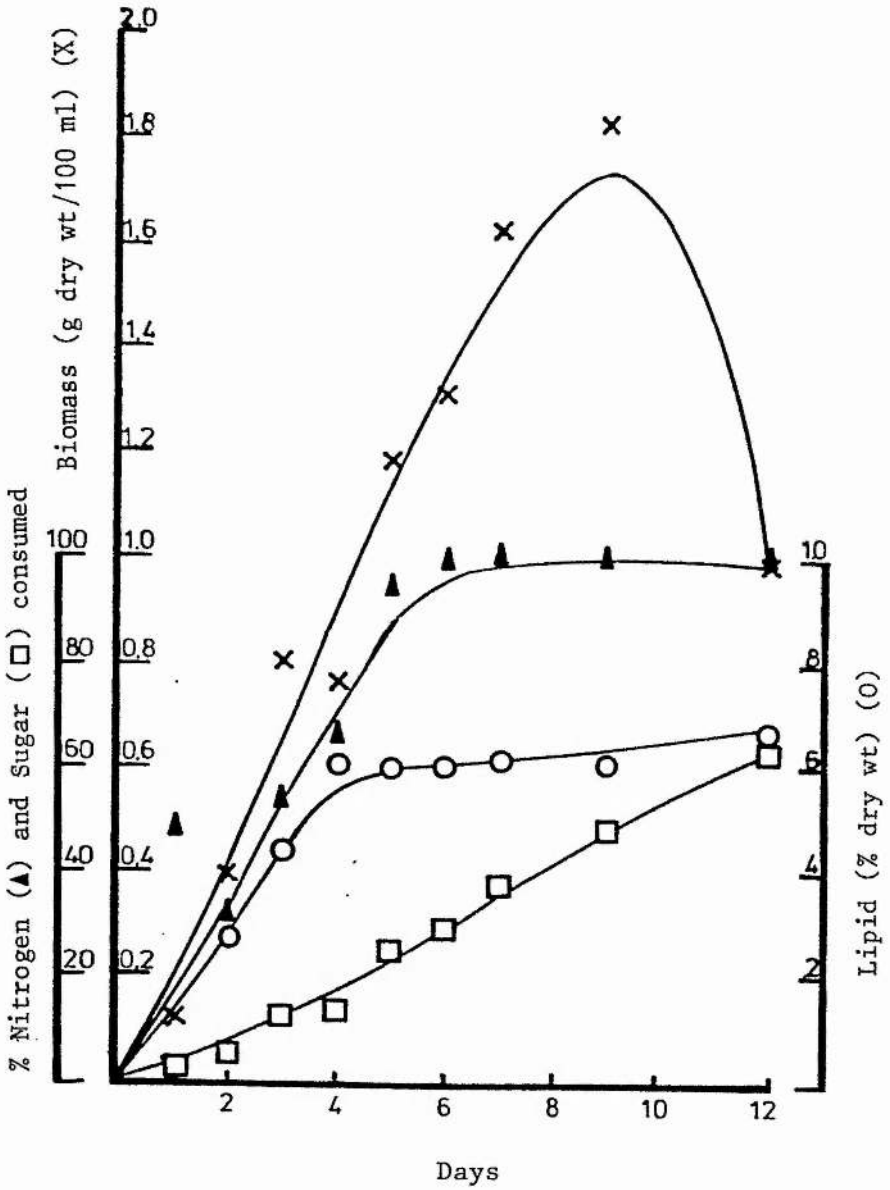


FIGURE 5. Biomass, nitrogen and sugar consumption and lipid content of wild type, strain *16*, of *A. nidulans* grown in N&S medium in fermenter, at 30°C.

2.4.3 Effect of method of culture/aeration

2.4.3.1 Unagitated culture

An experiment was carried out, with strain *16*, to investigate the influence of medium composition and sugar concentration on biomass formation and lipid accumulation. Three media were tested using 250 ml flasks containing 100 ml of each medium at 30°C. One flask, for each different medium, was harvested every two days and dry weight, lipid content, nitrogen and sugar consumption were determined. The results of this experiment are shown in Table 11 and illustrated in Figure 6.

The media used were:

a) N&S (17% glucose)

b) MMM (MM + 17% glucose)

c) modified N&S medium (5% glucose)

The important findings of this experiment are:

(i) MMM which has lower inorganic salt concentration than, but the same amount of glucose (17%) and $NaNO_3$ (6%) as N&S medium, gave better biomass production.

(ii) The growth rate is very low (due probably to the reduced aeration and low temperature). So that after 22 days some sugar and $NaNO_3$ are still left in the medium.

(iii) Comparing N&S medium with 17% glucose and with 5% glucose there is, as expected, a great difference in the biomass, but the percentage of lipid in the mycelium is almost the same. The nitrogen expires more quickly in the medium with 17% glucose than in that with 5%. Glucose also increases the growth rate.

Thereafter, experiments were carried out to investigate the effect of altering the $NaNO_3$ and phosphorus concentrations of MM, containing 12% glucose (i.e. altering C:N and C:P ratios), under four different conditions of cultivation: unagitated, orbital shaker, fermenter and vortex stirrer.

Days of incubation	Biomass (dry wt/100 ml)			Lipid (% dry wt)			% sugar consumed			% $NaNO_3$ consumed			Total lipid (mg/100 ml)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
4	0.07	0.32	0.23	-	4.92	5.44	11.10	33.60	32.30	11.19	25.69	16.29	-	15.74	12.51
6	0.81	0.85	0.20	5.00	5.80	6.63	22.16	24.82	35.04	30,95	42.58	10.37	40.50	49.30	13.26
8	1.14	0.98	0.52	5.44	4.60	5.87	26.12	30.69	32.77	60.30	43.85	24.44	62.02	45.08	30.37
10	0.62	1.64	0.61	4.00	4.64	5.20	18.47	36.40	43.55	71.17	24.44	35.95	24.80	75.93	31.72
12	1.30	2.21	0.73	9.76	8.36	8.70	34.24	49.10	49.25	66.14	94.32	37.10	126.88	184.76	63.51
14	1.82	3.19	1.03	5.40	7.60	5.96	59.71	63.79	84.04	99.07	100	58.95	98.28	242.44	61.39
16	2.07	2.61	1.21	4.68	7.68	5.48	51.94	62.48	79.48	92.16	100	45.72	96.88	200.45	66.31
18	3.75	2.79	0.97	8.80	8.04	5.92	80.31	66.41	79.43	100	100	53.20	330.00	223.51	57.42
20	1.38	3.08	1.10	5.40	11.84	5.80	43.85	74.82	97.92	100	100	69.10	74.52	367.67	63.80
22	2.40	3.51	1.10	6.68	11.48	6.28	79.07	81.72	97.43	100	100	72.18	160.32	402.95	69.08

1 = N&S medium; 2 = MMM (17% glucose); 3 = N&S medium (5% glucose).

TABLE 11. Biomass, lipid content, sugar and nitrogen consumption of *A. nidulans*, wild type *16*, grown in three different media in incubator, without agitation, at 30°C.

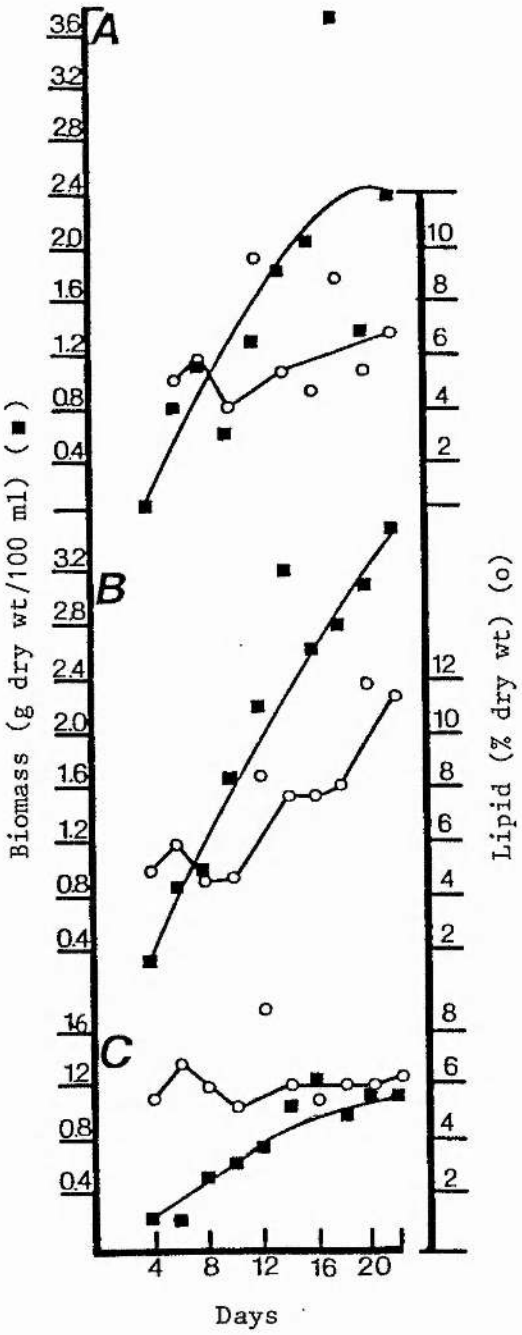


FIGURE 6. Biomass and lipid content of *A. nidulans*, strain *16*, grown in incubator without agitation at 30°C. A = N&S medium; B = MMM (17% glucose); C = N&S medium (5% glucose).

2.4.3.2 Unagitated and shaken cultures

To further investigate the influence of medium composition on lipid production, an experiment was carried out with wild type *16* in MMM with 12% glucose, 0.1 or 0.2% nitrogen source ($NaNO_3$) and an increased amount of phosphorus (KH_2PO_4), from 0.15 to 0.5%.

The mould was grown in the orbital shaker and incubator without agitation, at 30°C over a period of 20 days, taking one flask, for analysis, every two days. These results are shown in Table 12 and illustrated in Figure 7.

The important findings from this experiment are:

(i) Although making no difference to the biomass yield at the end of incubation period, growth in the shaker gave a two-fold increase in the lipid content compared with unshaken cultures, using identical medium.

(ii) 0.2% $NaNO_3$ resulted in twice the biomass yield obtained with 0.1% $NaNO_3$ in the shaken cultures.

(iii) 0.1% $NaNO_3$, however, produced a 40% improvement in lipid content in comparison with 0.2% $NaNO_3$.

(iv) Considering (ii) and (iii) together, however, the total lipid from the culture is highest with 0.2% $NaNO_3$.

(v) With both concentrations of $NaNO_3$ all the nitrogen was consumed by day 6 of culture, although sugar consumption continued after this. As expected, lipid content did not increase until after all the nitrogen had been consumed.

Days of incubation	Biomass (g dry wt/100 ml)			Lipid (% dry wt)			% sugar consumed			% $NaNO_3$ consumed			Total lipid (mg/100 ml)		
	0.1i	0.1s	0.2s	0.1i	0.1s	0.2s	0.1i	0.1s	0.2s	0.1i	0.1s	0.2s	0.1i	0.1s	0.2s
2	-	0.19	0.15	-	2.79	3.07	-	0.00	0.00	-	22.7	18.72	-	5.30	4.61
4	0.30	0.80	0.95	5.08	5.44	4.52	0.00	14.14	22.60	55.00	54.30	86.81	15.64	43.52	42.94
6	0.72	1.32	1.53	4.12	4.84	5.60	14.14	20.18	24.78	91.00	100	100	29.66	63.89	85.68
8	0.80	0.73	1.50	5.08	9.08	6.12	17.77	20.18	32.27	100	100	100	40.64	66.28	91.80
10	0.76	1.13	1.54	7.16	9.32	8.80	11.12	15.05	36.42	100	100	100	54.42	105.32	135.52
12	0.74	0.86	1.40	6.92	10.04	9.32	13.11	21.39	36.50	100	100	100	51.21	86.34	130.48
14	0.88	0.98	1.60	6.72	9.68	7.24	25.80	16.86	43.30	100	100	100	59.14	94.86	115.84
16	0.87	1.13	1.51	6.08	12.80	9.88	13.84	33.48	44.48	100	100	100	52.90	114.64	149.19
18	0.87	1.08	1.73	7.48	11.60	9.32	24.41	11.72	44.35	100	100	100	65.08	125.28	161.27
20	0.92	0.92	1.73	6.48	13.92	10.00	13.84	15.35	51.00	100	100	100	59.62	128.06	173.00

0.1 or 0.2 = % $NaNO_3$; i = incubator; s = shaker

TABLE 12. Biomass, lipid content, sugar and nitrogen consumption of strain *16* grown in MMM (12% glucose + 0.1 or 0.2 % $NaNO_3$ + 0.5% KH_2PO_4) in incubator, without agitation, and orbital shaker at 30°C.

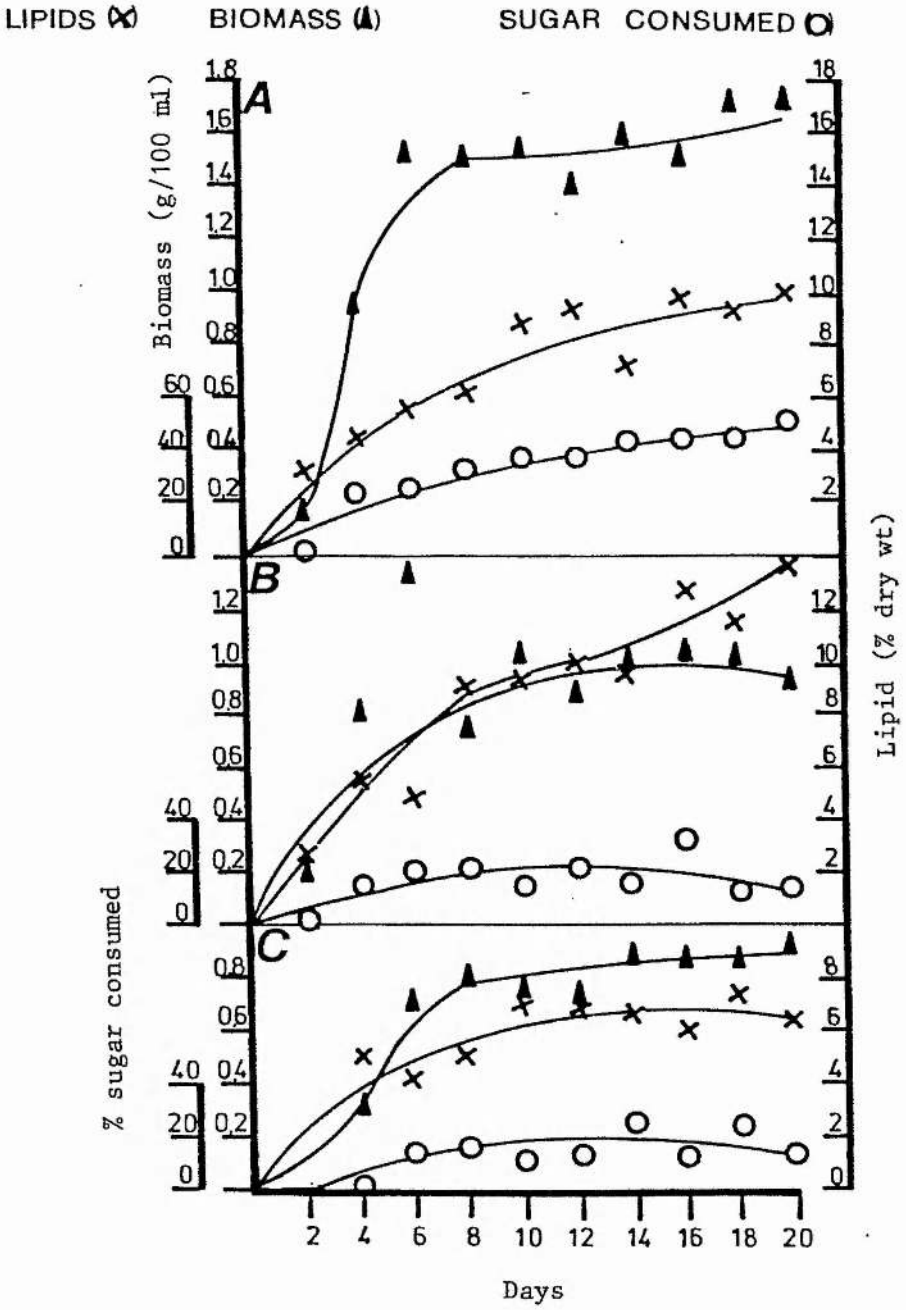


FIGURE 7. Biomass, lipid content and sugar consumption of wild type *16*, grown in different conditions. A = MMM (12% glucose + 0.2% $NaNO_3$ + 0.5% KH_2PO_4) in orbital shaker; B = MMM (as A, but 0.1% $NaNO_3$) in orbital shaker; C = (as B, but in incubator without agitation).

2.4.3.3 Fermenter culture

Having obtained a considerable improvement in lipid production with modified MM (12% glucose + 0.1% $NaNO_3$ + 0.5% KH_2PO_4) in shaken culture, the same strain, wild type *16*, was grown in the same medium in the fermenter at 30°C over a period of 18 days; harvesting 100 ml of sample daily for analyses.

The inoculum was made using 2 x 500 ml, 48 hour cultures grown in shaker at 30°C and consisting mainly of pellets. The subsequent growth in the fermenter was in the form of small pellets.

The results are shown in Table 13 and Figure 8. The lipid content of the fungus reached a maximum of 25 percent by the 10th day and then declined. This figure was over twice the previous best yield with the same medium. This must be due, primarily, both to the superior gas transfer obtained with the fermenter and to C:N ratio, since neither the same medium in the shaker (2.4.3.2) nor a different medium in the fermenter (2.4.2) produced as high a lipid yield.

Again, almost $^2/_3$ of the total dry mycelium obtained at the end of the incubation period was from that adhering to the glass vessel above the level of the culture media (5 l of medium were used in a 10 l jar). Because of this "wall growth" the biomass data, particularly the later points, are unreliable.

The nitrogen source expired rapidly (on the second day of incubation) indicating a very rapid period of cell growth which, unfortunately, could not be confirmed with any accuracy gravimetrically due to the problems caused by fermenter wall growth. From this data it is likely that cessation of growth is due to nitrogen exhaustion. Certainly the pH data show that a substantial change in pH is not the cause of entry into stationary phase.

The estimation of glucose consumption in these experiments, using

Days of incubation	Biomass (g dry wt/100 ml)	% sugar consumed DNSA*	% sugar consumed G.O.**	% $NaNO_3$ consumed	Lipid (% dry wt)	pH	Total lipid (mg/100 ml)
1	0.35	0.0	11.5	97.89	5.68	6.3	19.88
2	0.37	9.8	14.0	100	6.64	5.5	24.57
3	0.44	22.0	37.1	100	8.88	5.4	39.07
4	0.35	19.0	23.1	100	12.12	5.4	42.42
5	0.40	23.0	40.0	100	13.80	5.4	55.20
6	0.38	51.2	40.0	100	15.72	5.4	59.74
7	0.46	33.2	40.0	100	16.32	5.4	75.07
8	0.23	34.1	40.0	100	18.18	5.5	41.81
9	0.24	16.0	38.5	100	20.26	5.5	48.62
10	0.33	43.6	47.6	100	24.88	5.5	82.10
11	0.33	20.4	32.9	100	22.24	5.5	73.39
12	0.29	29.3	43.4	100	19.80	5.5	57.42
13	0.30	15.2	30.1	100	19.16	5.5	57.48
15	0.23	6.7	14.7	100	18.56	5.6	42.60
17	0.18	24.0	28.7	100	20.04	5.6	36.07
18	0.58***	14.4	32.9	100	17.00	5.6	219.30

*DNSA - DNSA method (Bruner, 1964)

**G.O. - glucose oxidase method (Updike and Hicks, 1967)

*** This figure is the mycelium weight remaining in the medium. However, much wall growth was also collected at this final harvest amounting to 26.2 g. Total biomass yield for 5 l (wall grown mycelium + mycelium from the medium) = 55.2 g ≡ 1.10 g/100 ml.

TABLE 13. Biomass, lipid content, glucose and nitrogen consumption of strain *16*, grown in 5 l of MMM in fermenter, at 30°C.

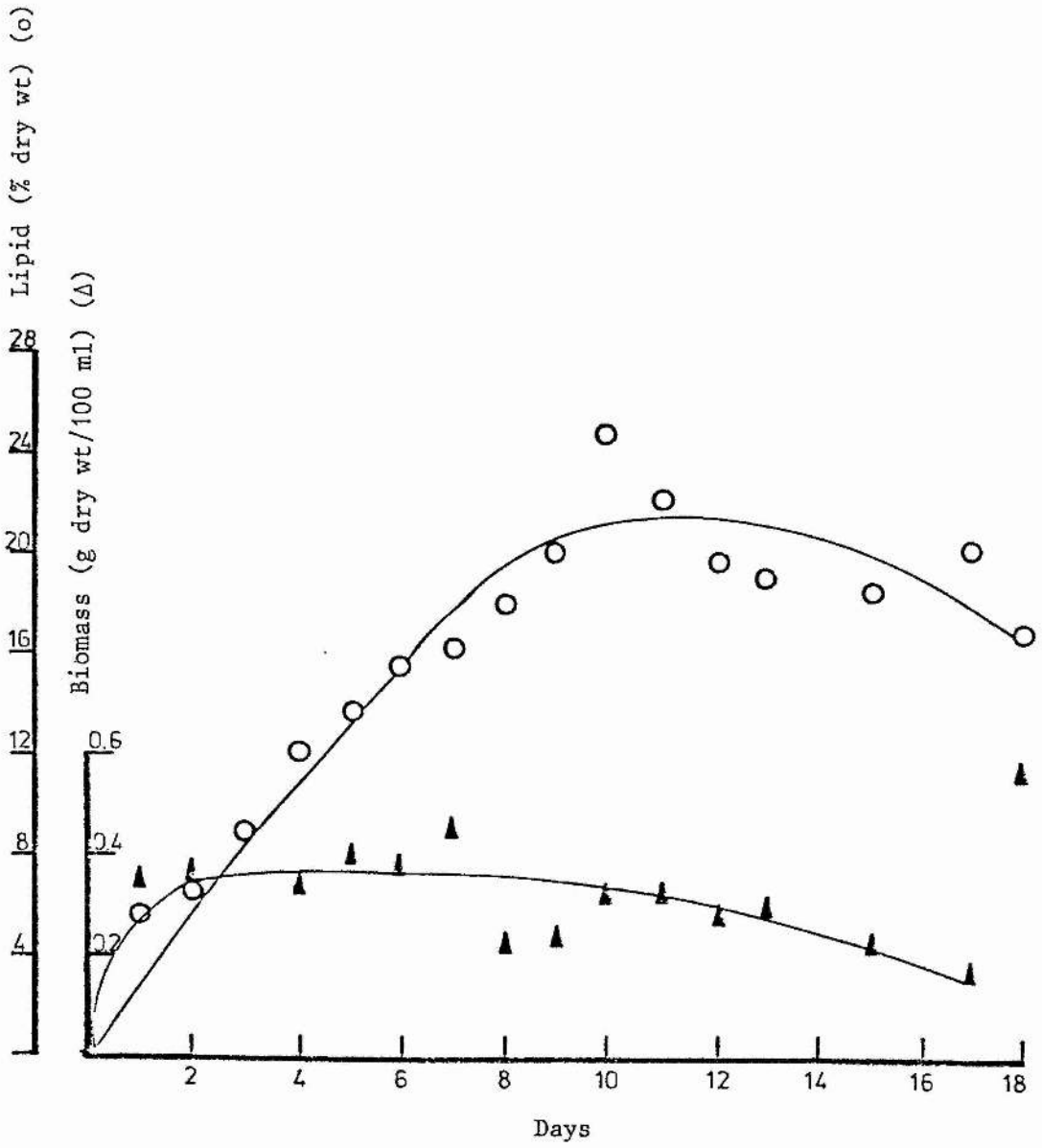


FIGURE 8. Biomass and lipid content of wild type *16*, grown in MMM (12% glucose + 0.1% $NaNO_3$ + 0.5% KH_2PO_4) in fermenter at 30°C.

MMM (12% glucose + 0.1 $NaNO_3$ and 5% KH_2PO_4), either in incubator, shaker or fermenter (Tables 12 and 13) gave rather erratic results with, in some cases, the amount of glucose in the medium decreasing, but then increasing again. In the fermenter, this was found both for the DNSA method for reducing sugars and for the more specific glucose-oxidase method. This may be due to either: production of interfering substances or to the release of glucose/reducing sugar from the mycelial mass by perhaps dead and dying cells.

It was also noticed that, in this medium, increased pigmentation of the mycelial mass, growth medium and lipid extracts, occurred during fungal growth. This phenomenon was attributed to the presence of a higher concentation of potassium dihydrogen phosphate in the medium.

The concentration of potassium dihydrogen phosphate was lowered in further experiments to avoid pigment formation by the fungi, because this certainly interfered with subsequent lipid analysis (see 3.2.2.2.2 and 3.3.2) and may be the cause of some of the interference in glucose assay described here.

2.4.3.4 Vortex stirred culture

Since fermenter growth in this medium (MM + 12% glucose + 1% $NaNO_3$ + 0.15% KH_2PO_4) was clearly superior to shaken cultures, another culture technique which gives extremely efficient gas transfer, the vortex stirrer, was tried. Strain *16* was grown in 100 ml of medium in the orbital shaker at 37°C for 48 h. After that time medium and pellets were transferred to 1,000 ml flasks containing 400 ml of the same medium. One whole flask was harvested every 3 days. Under these conditions the morphology of the fungus consisted of much more finely divided particles, possibly even single cells.

This technique gave the highest lipid content in the dried mycelium. It increased steadily up to a maximum of 24 percent at 9 days, after which it decreased. Nitrate was undetectable after 3 days, indicating very rapid assimilation under these culture conditions. pH fell slightly, but not sufficiently to be the possible cause of cessation of growth.

Biomass readings were reliable up to 12 days, but after this vessel wall growth occurred, making subsequent readings unreliable.

The results of these experiments are shown in Table 14 and Figure 9.

Days of incubation	Biomass (g dry wt/100 ml)	Lipid (% dry wt)	% sugar consumed	% $NaNO_3$ consumed	pH	Total lipid (mg/100 ml)
3	0.37	6.30	11.72	100	6.2	23.31
6	0.42	13.77	23.05	100	5.8	56.57
9	0.67	23.59	33.61	100	6.0	158.05
12	0.59	17.73	34.93	100	6.1	104.61
15*	0.69	14.11	28.33	100	5.7	97.36

* Wall growth mycelium

TABLE 14. Biomass, lipid content, sugar and nitrogen consumption of strain *16*, grown in 500 ml MMM in vortex stirrer apparatus, at 37°C.

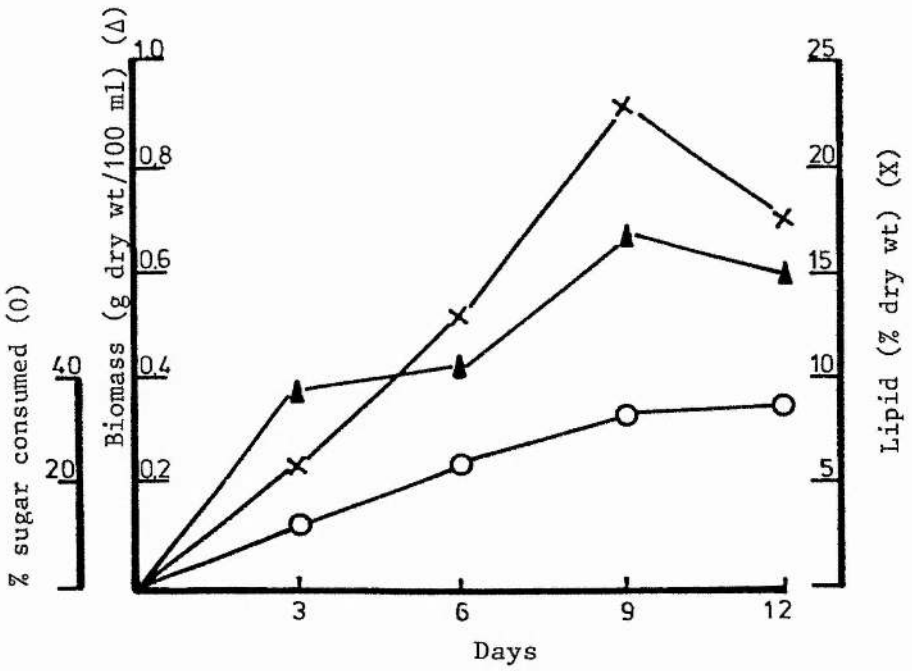


FIGURE 9. Biomass, lipid content and sugar consumption of strain *16* grown in MMM (12% glucose + 0.1% $NaNO_3$ + 0.15% KH_2PO_4) in vortex stirrer at 37°C.

3. Lipid analysis

3.1 Method development and selection

Naturally occurring lipids extractable with organic solvents are usually complex mixtures that can be easily separated by column chromatography into neutral and polar lipid fractions.

It was necessary to further resolve both the neutral lipids on TLC plates (to give: sterols, free fatty acids, triglycerides and sterol esters) and the polar lipids (into various phospholipids and glycolipid classes).

To obtain optimum resolution on TLC a variety of solvent combinations were tested with standards and *A. nidulans* lipids.

3.1.1 Neutral lipids TLC

Standard mixtures of neutral lipids were run in three different solvent systems on 0.25 mm layers of silica gel G:

a) benzene-ether-ethyl acetate-acetic acid. (80:10:10:0.2, v/v)

b) hexane-ether-formic acid. (80:20:2, v/v)

c) hexane-ether-acetic acid. (80:20:1, v/v)

System a. gave good separation of monoglyceride and diglycerides (including resolution of 1,2 and 1,3 isomers), but did not completely separate the groups: sterol/free fatty acid/diglyceride and triglycerides/ sterol esters.

Similar separations were achieved with both systems b. and c., which gave good resolation of all components. Both systems were considered as suitable and were used in subsequent experiments.

3.1.2 Polar lipids 2-D TLC

Four phospholipid standard mixtures were chromatographed on silica gel H in three different solvent systems with the aim of finding a system which could achieve optimum separation of all the components.

The first solvent system was that of Hunter *et al.* (1983), in the first dimension, and an acid mixture in the second:

1. $CHCl_3$-MeOH-NH_3 (25%, w/v) - H_2O. (160:88:11:11, v/v)
2. $CHCl_3$-MeOH-HAc (50:27:12, v/v).

This solvent system showed inadequate resolution of phosphatidylinositol (PI) and phosphatidylserine (PS).

The second solvent system was modified from Hunter *et al.* (1981) and Hunter *et al.* (1983) and used an acidic mixture in both dimensions:

1. $CHCl_3$-MeOH-HAc (50:27:12, v/v)
2. $CHCl_3$-MeOH-DIBK-HAc-H_2O (45:15:30:20:4, v/v).

Poor resolution of CL and PA was obtained with this solvent system.

The third solvent system used a combination of solvent system 1 and 2:

1. $CHCl_3$-MeOH-NH_3 (25% w/v) - H_2O (160:88:11:11, v/v)
2. $CHCl_3$-MeOH-DIBK-HAc-H_2O (45:15:30:20:4, v/v).

This solvent system gave best resolution of all four standard mixtures assayed and therefore was chosen for further separations. A map of the chromatogram obtained with the standard components, using this solvent system, is given in Figure 10.

Figure 11 shows a two dimensional TLC of total lipid of

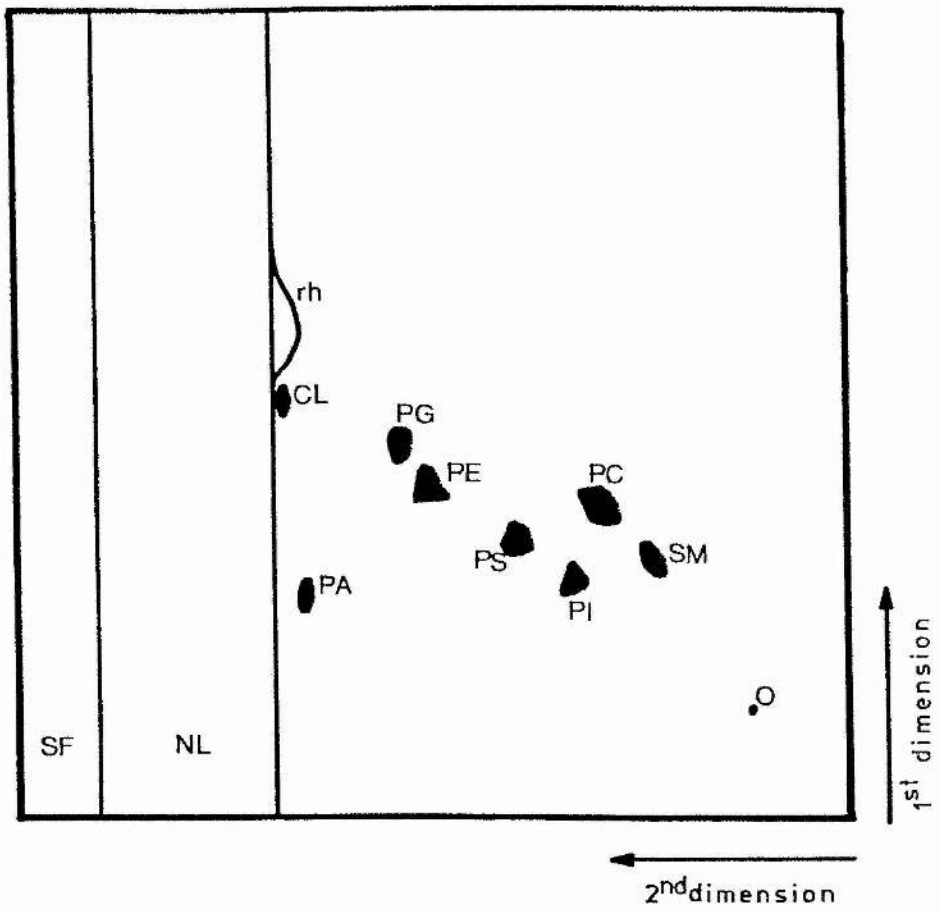


FIGURE 10. Two dimensional TLC separation of standard mixture on silica gel H.

Solvent system: 1st dimension: chloroform-methanol-ammonia (25%, w/v) - water. (160:88:11:11, v/v);

2nd dimension: chloroform-methanol-di iso butyl ketone-acetic acid-water. (45:15:30:20:4, v/v).

Abbreviations: 0 = origin; SM = sphingomyelin; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; CL = cardiolipin; PA = phosphatidic acid; rh = rhodamine; NL = neutral lipid; SF = solvent front.

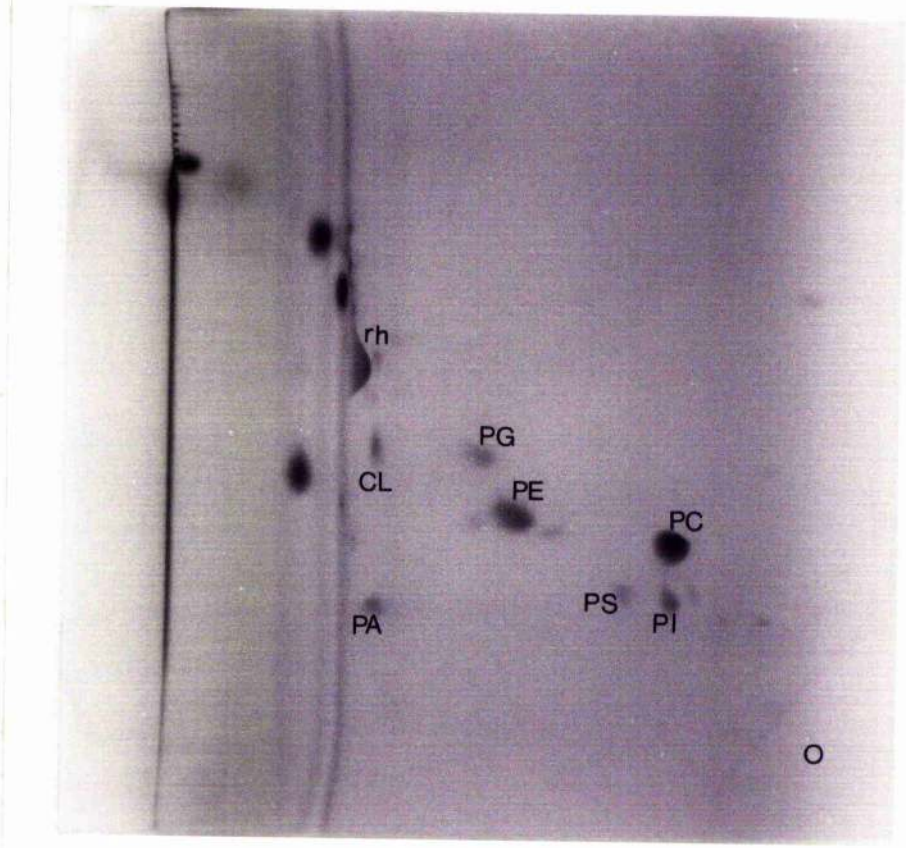


FIGURE 11. Two dimensional TLC of total lipid of strain *16* grown in N&S medium in incubator, at 30°C for 8 days.

Solvent system: 1: chloroform-methanol-ammonia (25%, w/v) - water. (160:88:11:11, v/v).

2: chloroform-methanol-di iso butyl ketone-acetic acid-water. (45:15:30:20:4, v/v).

Detection: iodine vapour.

Abbreviations: O = origin; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; CL = cardiolipin; PA = phosphatidic acid; rh = rhodamine.

A. nidulans, strain *16*, grown in incubator, without agitation, in N&S medium for 8 days at 30°C.

3.1.3 TLC detection reagent

Iodine vapour proved to be a very useful method and was the most used detection reagent, in this work, for either kind of lipids: neutral and polar. Lipids became visible as brown spots.

Rhodamine 6G was used as a non-destructive detection reagent and the lipids could be removed from the preparative plates for further analysis. Neutral lipids appeared as yellow and purple spots under ultraviolet light.

Sterol and sterol esters were specifically identified, using ferric chloride spray, as violet spots. Free fatty acids and triglycerides appeared as brown spots on a white background.

Concentrated sulphuric acid-glacial acetic acid (1:1, v/v) gave positive spots for all neutral lipid components, but did not differentiate sterols and sterol esters.

3.2 Lipid composition of *A. nidulans*, strain *16:* the influence of extraction method and growth medium.

A large scale lipid extraction was performed on 10 g of dry mycelium obtained from the fermenter after 18 days of culture at 30°C, in MMM (12% glucose + 0.1% $NaNO_3$ + 0.5% KH_2PO_4) (see section 2.4.3.3) and after 12 days of culture under the same conditions in N&S medium (see section 2.4.2).

Two lipid extraction methods were tested on cells grown in each of the above media and quantitative and qualitative analyses

were carried out, afterwards, using column chromatography, TLC and GLC. The first lipid extraction method involved treatment with hot isopropanol and is hereafter referred to as "isopropanol extraction method (IP). The second used chloroform-methanol (1:2, v/v) and is referred to subsequently as "chloroform-methanol extraction method" (CM).

3.2.1 Total lipid content

The total lipid content (g/100 g dry mycelium) was found to be:

Extraction method	Medium	
	MMM	N&S
IP	19.8	8.6
CM	22.5	6.8

These values are around those obtained in early experiments by extraction with CM, using only 0.25 g of dry mycelium (MMM = 17 percent and N&S = 6.7 percent - see Table 13 and Figure 10, respectively).

3.2.2 Lipid class fractionation on acid treated Florisil

3.2.2.1 Proportions of neutral lipids, glycolipids and phospholipids

The total lipids obtained by these two extraction methods, first were separated into three main classes by acid treated Florisil column chromatography. Each of these lipid classes were weighed and quantitative

analysis of lipid P carried out. The results are shown in Table 15. The corrected percentage of each fraction was calculated after adjustment for the phosphorus content of the glycolipid fraction.

The medium composition seems to have little effect on the proportion of each lipid class, although the total lipid content in MMM was greater (around three times) than in N&S (2.3.2.1).

On the other hand the extraction methods do influence the lipid class distribution for a given medium. In both media CM gives a higher proportion of phospholipids than IP. The probable reason for this is the ability of CM to extract more protein bound lipids from membranes, since methanol is a better protein denaturant than isopropanol.

Thus *A. nidulans*, strain *16*, lipids consist of about 75 percent neutral lipid, 8 percent glycolipid and 17 percent phospholipid, when extracted with CM and approximately 86 percent neutral lipid, with 7 percent glycolipid and only 7 percent phospholipid, when extracted with IP.

Phosphorus estimations of the fractions showed that the glycolipid fractions contained minor contamination with phospholipids. This is recognised as a problem (Kates, 1982) in natural lipid mixtures containing a high proportion of acidic phospholipids, some of which are eluted from the column with acetone.

The amount of phospholipid, in the fractions, can be calculated by multiplying % P by 25. It can be seen that the polar lipid fraction extracted by CM from N&S, contains almost 100% phospholipid by this calculation, but IP from N&S and both extraction method from MMM yield polar lipid fractions with much lower amounts of total phospholipid.

For cells grown in MMM, the lack of recovery of lipid P and the low percentage of P, in the phospholipid fraction (this should be around 4 percent for most known phospholipids) may be due to:

(i) large amounts of non lipid P extracted and left on the column

(ii) large amounts of non P containing polar lipids in the phospholipid fraction

Lipid class	Fraction proportion (% of total wt)											
	MMM						N&S medium					
	Extraction Method											
	IP			CM			IP			CM		
	% total	% P	% total corrected	% total	% P	% total corrected	% total	% P	% total corrected	% total	% P	% total corrected
NL	87.05 ± 2.73	0.000	87.05	76.03 ± 7.88	0.000	76.03	85.74 ± 1.34	0.000	85.74	73.84 ± 1.03	0.000	73.84
GL	5.21 ± 0.82	0.005	5.21	9.06 ± 1.85	0.058	8.93	7.98 ± 0.42	0.134	7.71	8.91 ± 0.33	0.880	6.95
PL	7.74 ± 3.44	0.244	7.74	14.92 ± 8.87	1.169	15.05	6.28 ± 0.91	2.923	6.55	17.26 ± 0.70	3.632	19.22
% recovery crude lipid	86.83			80.73			86.42			82.97		
% recovery of lipid P	45.80			65.10			90.67			85.03		

TABLE 15. Lipid class composition, after acid treated Florisil fractionation, of *A. nidulans*. grown in two different media and comparing two extraction methods. The results are mean of three separate fractionations for each sample and the standard deviation is shown beside the mean weight of each fraction. The percentage of phosphorus is the mean of duplicate estimations.

(iii) the presence of novel phospholipids with a much lower P content than those commonly found.

3.2.2.2 Monitoring of class fractionation by TLC

In addition to lipid P estimation, TLC was also used to monitor the neutral and polar lipid composition of these lipid fractions.

3.2.2.2.1 1-D TLC of neutral lipids

Figure 12 shows a silica gel G chromatogram of the fractionated and non-fractionated lipid of strain *16* grown in MMM, extracted by the two extraction methods. Authentic standards were also chromatographed.

It can be seen that the majority of the neutral lipids had eluted in the appropriate fraction obtained by Florisil chromatography. Very small traces of neutral lipids (sterol or DG?) can be seen in association with the glycolipid and phospholipid fractions. These traces could arise due to some neutral lipid bound to polar lipid and not eluted with NL on the column.

However, the NL fraction does contain some relatively polar lipid material which did not migrate from the origin. From the standards run, this must be either MG or material (containing e.g. 1 or more OH groups) which is as, or more, polar than MG.

The chromatogram shown in Figure 13 shows the same fractions as Figure 12, but with lipid from the fungi grown in N&S medium.

The major neutral lipid components found in cells grown in these two media were triglycerides (spot no. 3) and more polar materials which were later identified as sterol(s) (spot no. 1). Some traces of free fatty acids and sterol esters were also detectable.

The glycolipid fraction from the isopropanol extract contains a spot corresponding to triglycerides. The probable reason for its presence

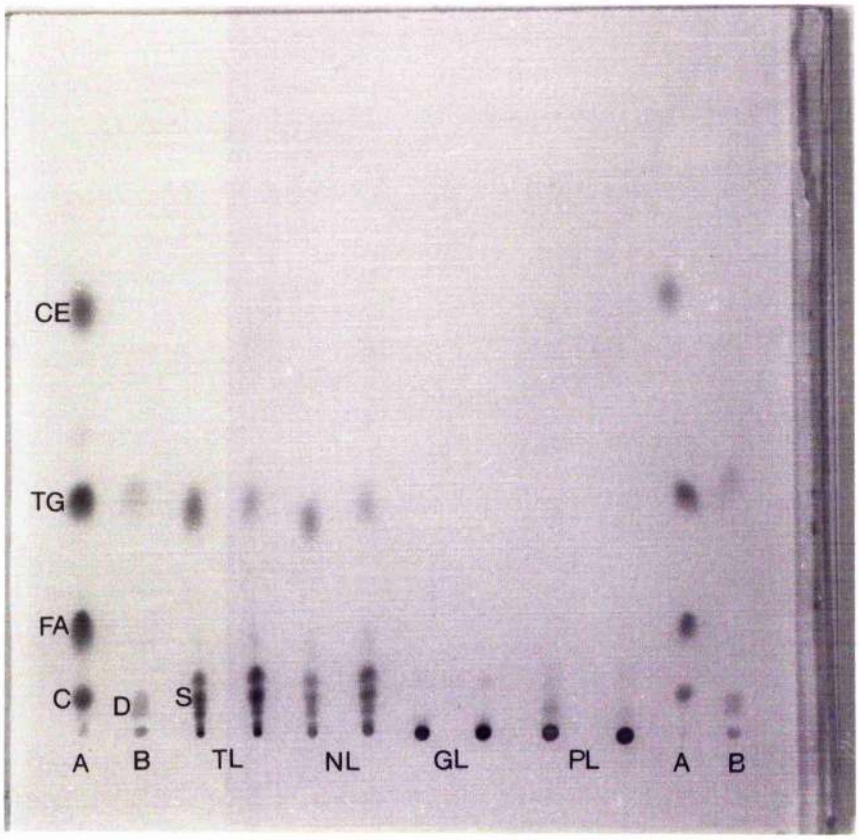


FIGURE 12. TLC separation on 0.25 mm layer of silica gel G, of fractionated and total lipid, of strain *16* grown in MMM in fermenter.

Solvent system: hexane-ether-acetic acid. (80:20:1, v/v).

Detection: iodine vapour.

Key to lanes: A and B = standards; TL = total lipid; NL = neutral lipid; GL = glycolipid; PL = phospholipid. (paired lanes = IP, CM).

Key to spots: C = cholesterol; FA = free fatty acid; TG = triglyceride; CE = cholesterol ester; D = diglyceride (1,2 and 1,3); S = sterols.

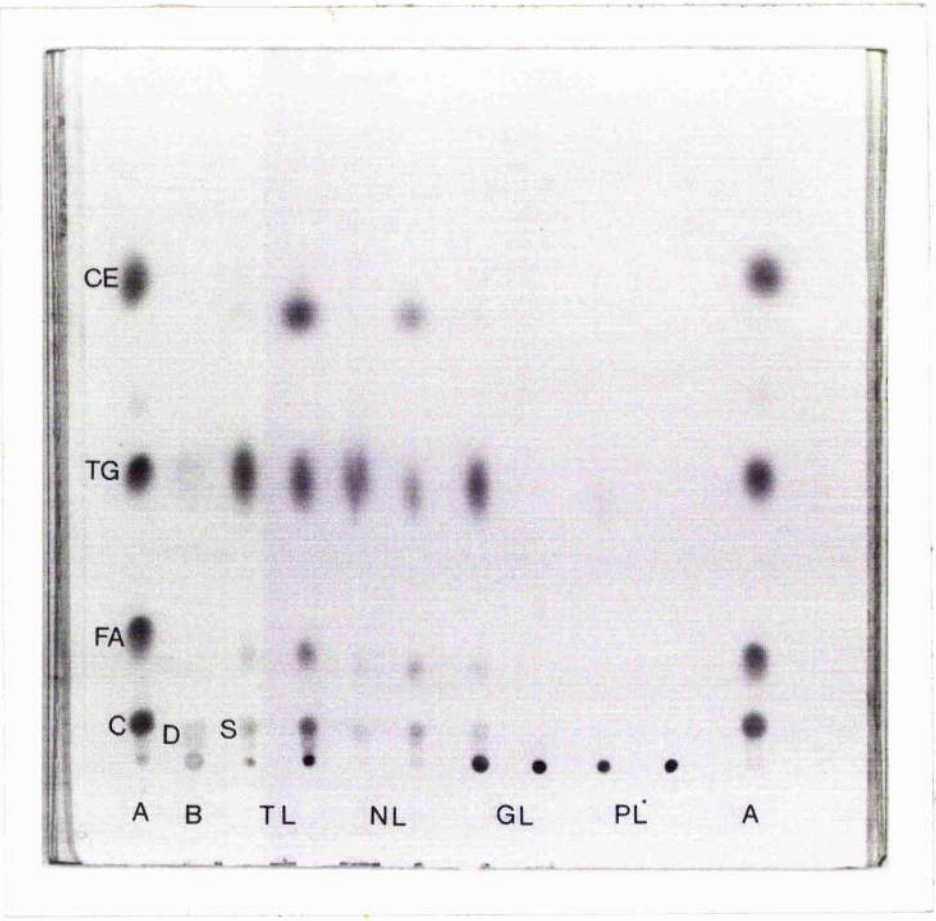


FIGURE 13. TLC separation on 0.25 mm layer of silica gel G, of fractionated and total lipid, of strain *16* grown in N&S medium in fermenter.

Solvent system: hexane-ether-acetic acid. (80:20:1. v/v).

Detection: iodine vapour.

Key to lanes: A and B = standards; TL = total lipid; NL = neutral lipid; GL = glycolipid; PL = phospholipid. (paired lanes = IP, CM).

Key to spots: C = cholesterol; FA = free fatty acid; TG = triglyceride; CE = cholesterol esters; D = diglyceride (1,2 and 1,3), S = sterol.

is that the column was overloaded, but it is a very small proportion of the total material.

A striking difference can be seen between the neutral lipid composition in N&S grown cells, which have a high proportion of a component later identified as sterol esters (SE - Figure 13) and MMM grown cells which contain only traces of sterol esters.

The different composition of the neutral lipid fraction found with the two media is important for any industrial process in that TG are desirable and sterol esters probably not.

Another important aspect seen clearly in Figure 13 is the superior ability of CM to extract sterol esters which may indicate protein binding of the latter..

3.2.2.2.2 1-D TLC of polar lipids

Silica gel H chromatograms of the fractionated and non-fractionated lipid of strain *16* show (Figures 14 and 15) that there is no polar lipid contaminating the neutral lipid fractions, but confirm the polar lipid fractions contain minor contamination with neutral lipids. These chromatograms also indicate that the more polar components left at the origin in Figures 12 and 13, in the NL fractions, are genuinely neutral lipid components, not polar lipid contaminants.

These preliminary 1-D TLC separations also allowed the tentative identification of PC and PE as the major polar lipids with PI as minor component.

An unexplained and unresolved problem was that the total lipid and phospholipid fractions, extracted by either method from MMM grown cells gave bad streaking and only one clearly visible component (PC). This may be due to interference from the large amounts of pigments which were produced in this particular medium.

Although the medium composition did not affect the proportions of

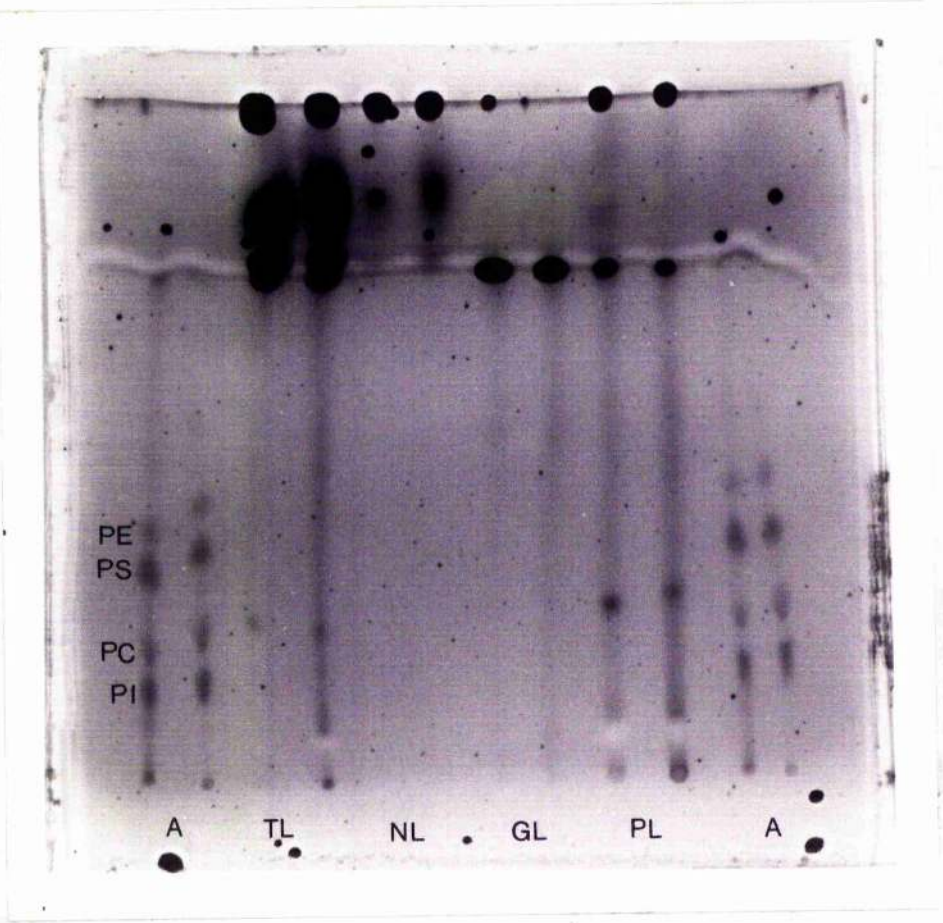


FIGURE 14. TLC separation on silica gel H, of fractionated and total lipid, of strain *16*, grown in MMM in fermenter.

Solvent system: chloroform-methanol-di iso butyl ketone-acetic acid-water. (45:15:30:20:4, v/v).

Detection: iodine vapour.

Key to lanes: A = standard; TL = total lipid; NL = neutral lipid; GL = glycolipid; PL = phospholipid. (paired lanes = IP, CM).

Key to spots: PI = phosphatidylinositol; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine.

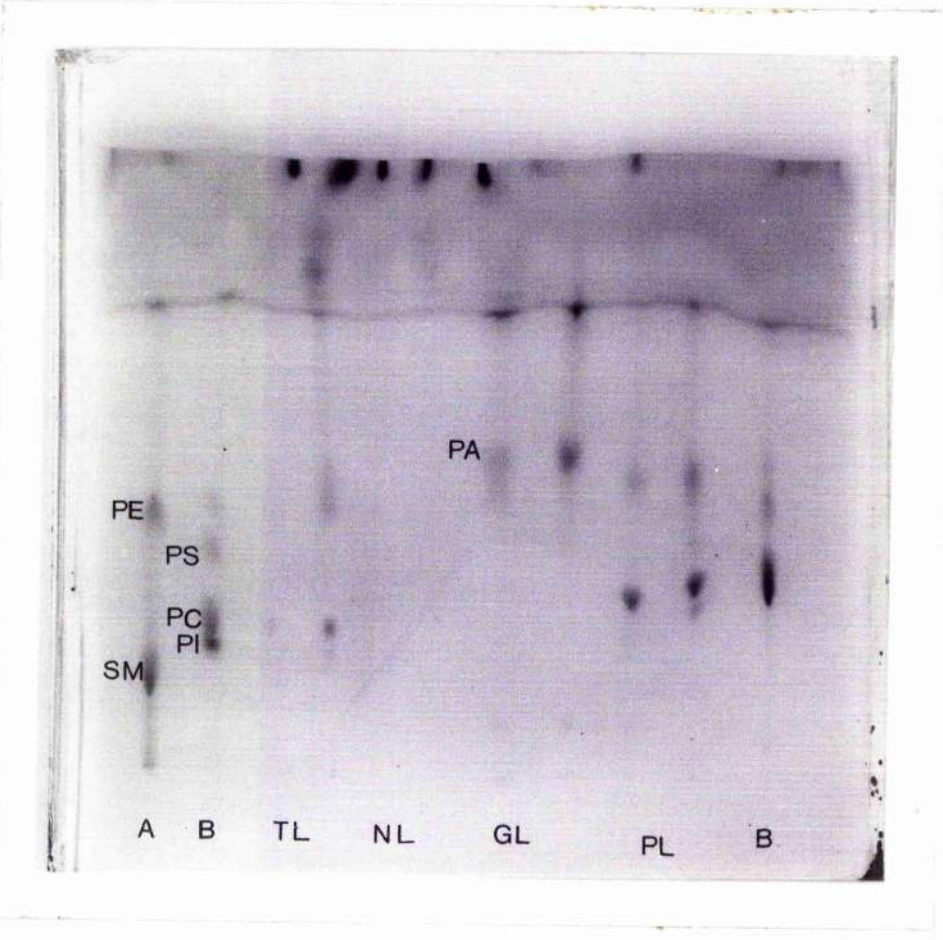


FIGURE 15. TLC separation on silica gel H of fractionated and total lipid of strain *16* grown in N&S medium in fermenter.

Solvent system: chloroform-methanol-di iso butyl ketone-acetic acid-water. (45:15:30:20:4, v/v).

Detection: iodine vapour.

Key to lanes: A and B = stnadards; TL = total lipid; NL = neutral lipid; GL = glycolipid; PL = phospholipid. (paired lanes = IP, CM).

Key to spots: CM = sphingomyelin; PI = phosphatidylinositol; PC = phosphatidylcholine; PS = phosphatidylserine; PE = phosphatidylethanolamine.

total neutral and polar lipids (Table 15), the composition of polar lipid classes was clearly affected, as can be seen when comparing Figures 14 and 15.

3.2.2.3 Fatty acid composition of total lipid and lipid classes

Table 16 shows tentatively identified major fatty acids (16:0, 18:0, 18:1, 18:2) of fractionated and unfractionated lipid of strain *16* grown in MMM and N&S medium, obtained by the two extraction methods.

The fatty acid composition of fractionated and unfractionated does differ slightly between the two extraction methods. In particular, the total lipid and neutral lipid contain a higher proportion of 17:0 after CM extraction.

The two media did not seem to significantly affect the fatty acid composition of unfractionated lipid, but marked differences can be seen for the glyco- and phospholipids, mainly for 18:2.

Polyunsaturates and/or long chain fatty acids appear in greater proportion in the glyco- and phospholipids than in neutral lipids and a greater proportion of these fatty acids are found in MMM compared with N&S medium.

3.2.2.4 Further fractionation of neutral lipid into sub-classes

3.2.2.4.1 Florisil column chromatography

The neutral lipid fraction was further separated into sub-fractions on 7 percent hydrated Florisil by column chromatography. Weights were determined for each fraction and are shown in Table 17.

The results for MMM are for duplicate chromatography separations, but for N&S medium are for one run only.

The quantitative neutral lipid sub-class composition differs for the two extraction methods only in N&S medium where CM extracted a bigger proportion of sterol and sterol esters. A lower recovery is observed

Fractions	Medium	Extraction method	FA (% of total acids)											
			16:0	17:0	18:0	18:1	18:2(?)	18:2	21.6*	21.8*	22:0	22.8*	22:2	22:4
TL	MMM	IP	21.6	T	20.9	22.2	T	35.3	T	T	T	T	T	-
		CM	21.4	5.2	18.2	20.4	T	34.8	T	T	T	T	T	-
	N&S	IP	19.8	T	22.0	22.6	-	35.6	T	-	-	-	-	T
		CM	25.9	11.3	17.0	16.1	T	29.6	T	T	T	-	T	T
NL	MMM	IP	29.8	T	28.8	20.0	T	21.3	T	T	T	T	-	-
		CM	32.5	13.0	27.1	15.6	T	11.9	T	T	T	-	-	T
	N&S	IP	17.8	T	19.9	23.5	-	38.9	T	-	-	-	-	T
		CM	17.2	11.5	15.2	19.1	-	35.1	T	T	T	-	-	T
GL	MMM	IP	18.2	T	14.2	11.0	T	6.2	8.8	17.2	-	8.5	15.84	-
		CM	20.1	T	11.4	13.6	T	18.0	5.0	12.2	-	5.5	14.32	-
	N&S	IP	23.8	T	22.9	20.5	-	32.8	T	T	-	-	T	T
		CM	25.5	T	7.5	12.2	T	31.3	T	5.8	-	-	11.19	6.51
PL	MMM	IP	37.2	6.13	16.3	12.1	T	7.6	10.03	-	10.7	T	-	-
		CM	30.4	T	10.8	10.9	5.72	25.6	T	5.5	5.8	5.34	T	-
	N&S	IP	35.0	T	14.6	17.0	T	33.3	T	T	T	-	T	T
		CM	39.2	T	5.4	13.0	T	42.8	T	T	T	T	T	T

Notes: T = traces (< 5%)
- = not detected
* = carbon number of non-identified fatty acids
(1) = individual peak area/Σ peak areas x 100

TABLE 16. Fatty acid composition(1) of fractionated and unfractionated lipid of strain *15* grown in MMM and N&S medium.

Fraction No.	Components eluted Kates (1982)	TLC	Composition (% of total) MMM IP	MMM CM	N&S IP	N&S CM
1	HC	SE	6.59 ± 1.71	8.28 ± 0.28	2.82	11.69
2	SE	TG,FFA	27.98 ± 19.7	23.25 ± 0.25	87.26	64.10
3	TG	St	42.83 ± 13.0	40.53 ± 0.33	4.28	9.01
4	St	St	9.00 ± 3.12	8.05 ± 0.11	2.89	8.07
5	DG	?	7.77 ± 0.81	8.21 ± 0.89	1.43	3.41
6	MG	?	4.78 ± 1.30	4.38 ± 0.20	1.31	2.78
7	FFA	?	1.06 ± 0.27	1.32 ± 0.91	-	0.94
% Recovery			96.53	91.20	87.53	98.09

Abbreviations: HC = hydrocarbons; SE = steryl esters; TG = trigycerides; FFA = free fatty acids; St = sterol; DG = diglyceride; MG = monoglyceride.

TABLE 17. Neutral lipid sub-class composition of strain *16*, after column chromatography on 7% hydrated Florisil.

with isporopanol for N&S medium due to accidental loss of fraction 7 during the solvent evaporation step.

In MMM the major fraction is fraction 3, identified by comparation with standards on TLC as sterol, with triglyceride (fraction 2) as the second major fraction. On the other hand, on N&S medium, triglyceride appears as the major fraction.

The difference obtained between the two cultural media is possibly due not only to the media composition, but perhaps also other factors such as different age and stage of development of the mycelial mass.

3.2.2.4.2 Monitoring of NL sub-class separation by TLC

The identification of the column fractions was by TLC in comparison with authentic standards.

Figure 16 shows fractions of lipid extracted with IP and CM of strain *16* grown on MMM.

Figure 17 shows fractions of lipid extracted with IP and CM of strain *16* grown on N&S medium. The components of fraction 7 (isopropanol extraction), are actually artefacts derived from contamination of the sample during concentration.

TLC showed that the column fractionation had been largely successful i.e. three major sub-classes resolved: sterol ester, triglyceride and sterol.

The sterol fractions show a different pattern in Figures 16 and 17. Three different sterol components can be visualised in Figure 16, but only two major sterols are seen in Figure 17.

Fractions 5 and 6 were expected to be diglycerides and monoglycerides, respectively, but comparing with standards, in Figures 16 and 17, authentic diglycerides do not correspond to the spot in fraction 5, which is evidently a more polar material. Monoglycerides and diglycerides, if present, are therefore in very small quantity, and not detectable by TLC at these loadings.

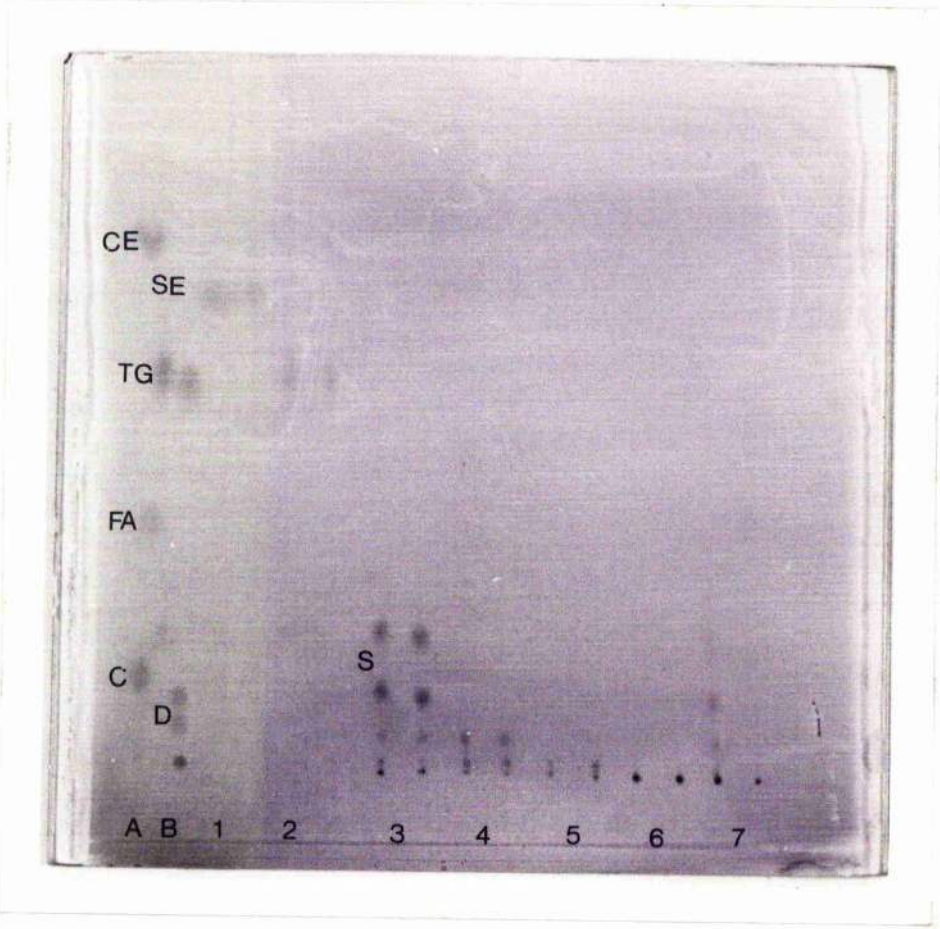


FIGURE 16. TLC separation on 0.25 mm layer of silica gel G of fractionated neutral lipid of strain *16* grown in MMM.

Solvent system: hexane-ether-formic acid. (80:20:2, v/v).

Detection: iodine vapour.

Key to lanes: A and B = standards; 1 to 7 = fraction number. (paired lanes = IP, CM).

Key to spots: C = cholesterol; FA = free fatty acid; TG = triglyceride; CE = cholesterol ester; D = diglyceride (1,2 and 1,3); SE = sterol ester; S = sterol.

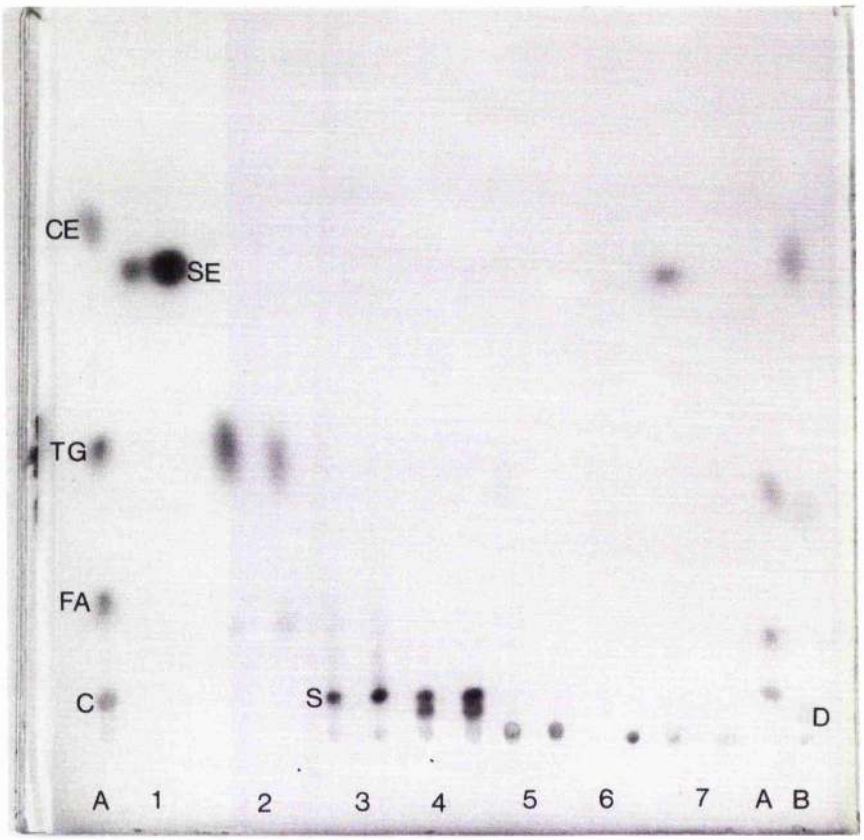


FIGURE 17. TLC separation on 0.25 mm layer of silica gel G of fractionated neutral lipid of strain *16* grown in N&S medium.

Solvent system: hexane-ether-acetic acid. (80:20:1, v/v).

Detection: iodine vapour.

Key to lanes: A and B = standards; 1 to 7 = fraction number. (paired lanes = IP, CM).

Key to spots: C = cholesterol; FA = free fatty acid; TG = triglyceride; CE = cholesterol ester; S = sterol; DG = diglyceride (1,2 and 1,3); SE = sterol ester.

Fractions 5, 6 and 7 contain relatively very small amounts of material which could not be identified by TLC.

Traces of free fatty acids were detected in fraction 2 in both Figures 16 and 17. Fatty acids do not occur as major constituents of lipid extracts (Gurr and James, 1975), and are usually artefacts due to lipase or phospholipase A activity on acyl lipids (Kates, 1982).

3.2.2.4.3 Fatty acid composition of neutral lipid sub-classes

Table 18 shows a comparison of fatty acid composition of triglyceride (fraction 2) with the total neutral lipid fraction of strain *16* grown in MMM and N&S medium, extracted with IP and CM.

The fatty acid composition of the triglyceride shows that the major fatty acids present were 16:0 (17 percent), 18:1 (22 percent) and 18:2 (38 percent). The proportion of these fatty acids did not differ significantly with extraction method or medium composition, except that the proportions of 18:2 were slightly higher in N&S medium and 17:0 constituted greated than 5 percent in MMM.

Comparing the triglyceride with the total neutral lipid some comments can be made:

(i) In both fractions, 17:0 is more abundant after CM extractions, in either medium, than after IP. This may be because it is esterified to a lipid (sterol ester) relatively tightly bound to protein, and methanol is a better protein denaturant than isopropanol.

(ii) The proportion of the triglyceride fatty acids in N&S medium is very much in accordance with that found in the neutral lipid fraction, but this is not true for MMM. This can be understood by looking back to Table 17, which shows different proportions of neutral components for MMM and N&S medium. Triglyceride is the major component on N&S medium, but in MMM other fatty acid containing fractions (sterol ester, diglyceride, monoglyceride) are in greater

Conditions			Fatty acids (% of total)				
			16:0	17:0	18:0	18:1	18:2
TG	MMM	IP	20.4	7.1	19.2	20.9	32.4
		CM	17.7	11.0	15.7	19.6	36.0
	N&S	IP	17.7	T	18.0	24.0	40.4
		CM	17.4	T	16.2	22.9	43.5
NL	MMM	IP	29.8	T	28.8	20.0	21.3
		CM	32.5	12.95	27.1	15.6	11.9
	N&S	IP	17.8	T	19.9	23.5	38.9
		CM	17.2	11.45	15.2	19.1	35.1

T = traces (<5 percent of total)

TABLE 18. Comparison of fatty acid composition of triglyceride (fraction 2) with neutral lipid fraction (data from Table 16) of strain *16* grown in MMM and N&S medium, extracted with IP and CM.

proportions.

The fatty acid composition, was also determined for all other neutral lipid fractions from CM extractions of strain *16* grown in N&S medium. Sterol ester (fraction 1) showed the presence of 17:0 as the major component and the other fractions contained only small traces of fatty acids.

3.3. Qualitative and quantitative analysis of polar lipids

3.3.1 Qualitative analysis and differential staining

The major phospholipids of *A. nidulans*, strain *16*, had already been tentatively identified from their mobilities on 1-D TLC as PC, PE and PI. To further confirm their identities, replicate 1-D plates of total lipid, were run and stained for:

(i) phosphorus

(ii) primary amino groups (ninhydrin)

(iii) carbohydrate (anthrone) and

(iv) charge (rhodamine).

Figure 18 shows strain *16* lipid run at four loadings together with total human erythrocyte lipid as standard, on silica gel H. Detection was with phosphorus reagent. SM, PI, PC, PS and PA appeared as blue spots and PE, PG and CL as grey spots. This reagent permitted detection in *A. nidulans*, strain *16* of: PI, PC, PS, PE (PG?), PA and CL.

Ninhydrin positive purple spots confirmed the presence of PS and PE (Figure 19).

No component of the total lipid, when sprayed with anthrone, gave a positive reaction for glycolipids, which suggests that, if present at all, there must only be small amounts.

Rhodamine staining gave purple/blue spots, seen under U.V. light, for acidic phospholipids (CL, PG, PS, PI, PA), whilst neutral lipids (PC, PE) gave yellow/pink spots.

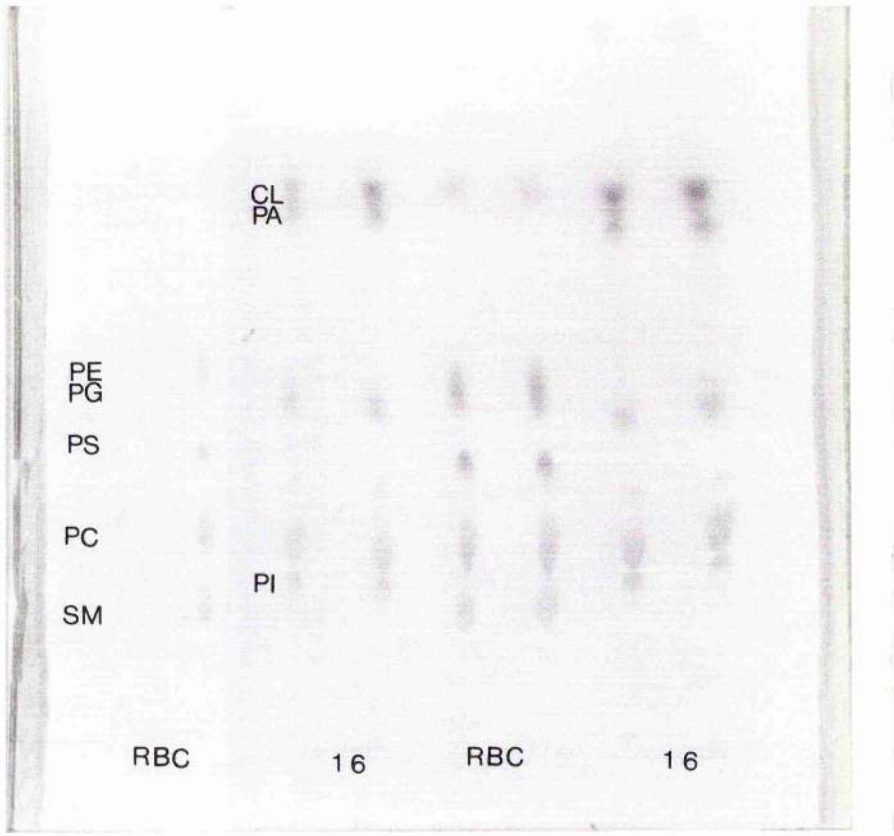


FIGURE 18. TLC separation on 0.25 mm layer of silica gel H of total human erythrocyte lipid (RBC) and total lipid of strain *16* grown in N&S medium.

Solvent system: chloroform-methanol-di iso butyl ketone-acetic acid-water. (45:15:30:20:4, v/v).

Detection: phosphorus stain reagent.

Key to lanes: RBC = Human erythrocyte total lipid, 16 = strain *16*.

Key to spots: SM = sphingomyelin; PI = phosphatidylinositol; PC = phosphatidylcoline; PS = phosphatidylserine; PE = phosphatidylethanolamine, PA = phosphatidic acid; CL = cardiolipin.

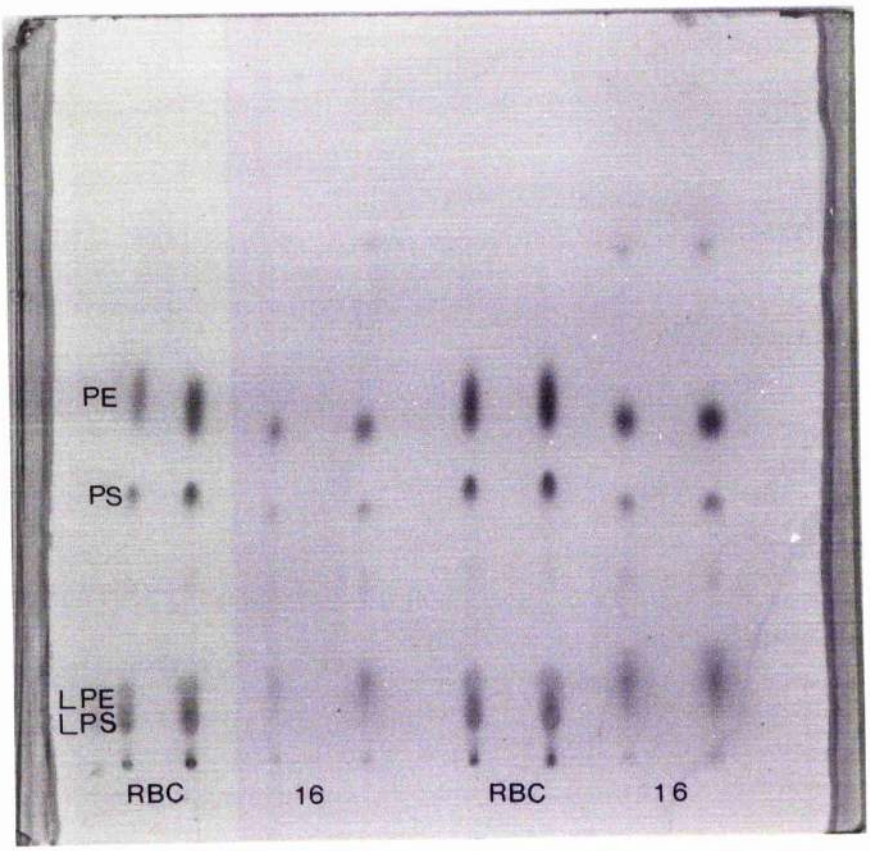


FIGURE 19. TLC separation on 0.25 mm layer of silica gel H of total human erythrocyte lipid (RBC) and total lipid strain *16* grown in N&S medium.

Solvent system: chloroform-methanol-di iso butyl ketone-acetic acid. (45:15:30:20:4, v/v).

Detection: ninhydrin.

Key to lanes: RBC = total human erythrocyte lipid; 16 = strain *16*.

Key to spots: LPE = lyso phosphatidylethanolamine; LPS = lyso phosphatidylserine; PE = phosphatidylethanolamine; PS = phosphatidylserine.

3.3.2 2-D TLC and quantitative analysis

Two dimensional TLC was performed on the glycolipid and phospholipid fractions of strain *16*, grown in MMM and N&S medium, obtained by both extraction methods (IP and CM).

The resolution of components obtained from MMM growth, was very poor due to much streaking of the chromatogram interfering with identification (see also 3.2.2.2.2). A probable explanation for this fact is the presence of large amounts of pigments in these fractions. These pigments are either strongly bound to, or have similar polarity to, glycolipids and phospholipids and are therefore eluted in the same fractions on column chromatography.

The glycolipid fractions from cells grown in N&S medium shows the same spots for both lipid extraction methods (Figures 20 and 21). Although further analysis was not carried out, it is possible to speculate from this relative chromatographic mobilities of plant glycolipids in similar solvent system (Hetherington, 1983) that the two glycolipid components observed here were monoglycosyl diglyceride (MGDG) and diglycosyl diglyceride (DGDG).

It is also interesting that, from qualitative examination of Figures 20 and 21, the ratio of DGDG/MGDG is much higher with CM than with IP extraction.

Phosphorus estimation was performed for each spot; the results are shown in Table 19. The absence of P from the spots tentatively identified as glycosyl diglycerides strengthened this identifaction. One spot was tentatively identified as phosphatidic acid (PA), and its P content also corroborated this assignment. Phosphorus estimation had already shown the glycolipid fraction (Table 15) to be contaminated by phospholipid. Two dimensional TLC now allowed the identification and quantitation of this phospholipid contaminant as PA. This enabled a further check to be made on the correction factor already applied to the lipid class fractionation for phospholipid contamination in the glycolipid fractions. The results

Spots	% P	
	IP	CM
MGDG	zero	zero
DGDG	zero	zero
PA	0.176	0.736
O	zero	zero

Abbreviations: MGDG = monoglycosyl diglyceride

DGDG = diglycosyl diglyceride

PA = phosphatidic acid

O = origin

%P = gP/100g total glycolipid fraction

(mean of two repetition)

TABLE 19. Phosphorus content of components of glycolipid fractions of strain *16* grown in N&S medium, IP and CM extractions, determined after 2-D TLC.

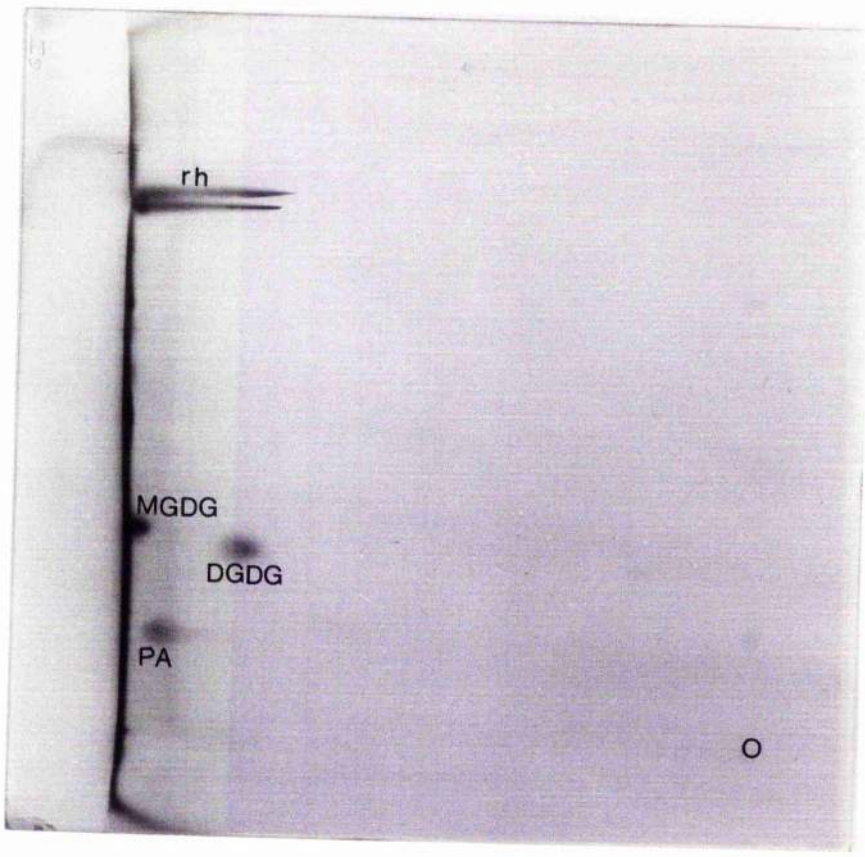


FIGURE 20. Two dimensional TLC of glycolipid fraction of strain *16*, grown in N&S medium, IP extraction.

Solvent system: 1. chloroform-methanol-ammonia (25%,w/v)-water. (160:88:11:11, v/v).

2. chloroform-methanol-acetic acid. (50:27:12, v/v).

Detection: iodine vapour.

Key to spots: 0 = origin; PA = phosphatidic acid, MGDG = monoglycosyl diglyceride; DGDG = diglycosyl diglyceride; rh = rhodamine.

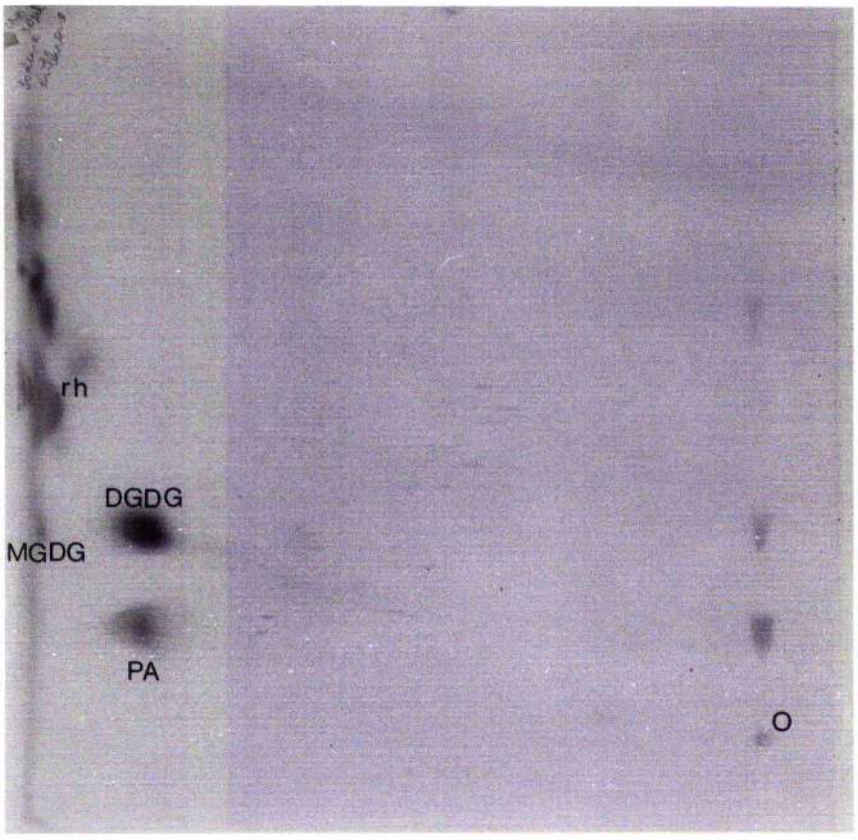


FIGURE 21. Two dimensional TLC of glycolipid fraction of strain *16*, grown in N&S medium, CM extraction.

Solvent system: 1. chloroform-methanol-ammonia (25%, w/v)-water. (160:88:11:11, v/v).

2. chloroform-methanol-acetic acid. (50:27:12, v/v).

Detection: anthrone (MGDG, DGDG - appeared purple, PA - yellow) and iodine vapour.

Key to spots: 0 = origin; PA = phosphatidic acid; MGDG = monoglycosyl diglyceride, DGDG = diglycosyl diglyceride; rh = rhodamine.

showed that the phosphorus contamination of the glycolipid fraction was due almost entirely to PA.

With this information it was also possible to correct the phospholipid composition shown in Table 20, to take account of the extra PA in the glycolipid fraction.

In contrast to the constant glycolipid pattern the phospholipid fractions showed different numbers of spots depending on the extraction method. Extractions with isopropanol gave only two major spots, identified as PC and PE + PG with traces of PA (Figure 22). Whilst after CM extraction PC, PI, PS, PE + PG, PA and CL were clearly identifable, although PC and PE were still the most abundant components (Figure 23). Quantitation of each component was determined from phosphorus estimation and the results are shown in Table 20.

The efficiency of extraction of phospholipid classes from lyophilized dried mycelium was clearly greater with CM than with IP.

The use of hot isopropanol is recommended by Kates (1982) for photosynthetic tissue containing rather stable degradative enzymes, in particular, phospholipase D, which may be released to attack phospholipids and cause an accumulation of phosphatidic acid and related compounds (Christie, 1982).

It is clearly likely, from the data in Table 20, that the use of isopropanol does indeed inhibit a phospholipase D, which results in the production of large amounts of PA in the CM method. It is also significant from the much lower PC value with CM that it is this phospholipid class, specifically, which is preferentially degraded.

Components	Phospholipids (% of total) IP	% corrected**	CM	% corrected**
PC	71.53 ± 2.46	68.4	39.89 ± 2.11	32.1
PI	T		10.81 ± 0.90	8.7
PS	T		1.81 ± 0.48	1.5
PE + PG*	26.48 ± 0.74	25.3	25.85 ± 1.50	20.8
CL	T		2.80 ± 0.91	2.3
PA	2.00 ± 0.99	6.3	18.86 ± 1.42	34.6
0	T		T	
% recovery of P after TLC	84.51		90.00	

T = traces, detectable by I_2 vapour, but below detection limit of colorimetric P assay (less than 0.30% total lipid P)

* because of the heavy loading necessary for quantitative analysis, the resolution between PE and PG was poor and necessitated their combination. However, it is apparent from inspection of less heavily loaded chromatogram (Figure 11) that PE is a major and PG a very minor component

** corrected composition allows for the PA present in the glycolipid fraction

TABLE 20. Phospholipid composition of *A. nidulans*, strain *16*, grown in N&S medium, IP and CM extraction, determined from lipid P estimation after 2-D TLC (expressed as percentage phosphorus (W/w).

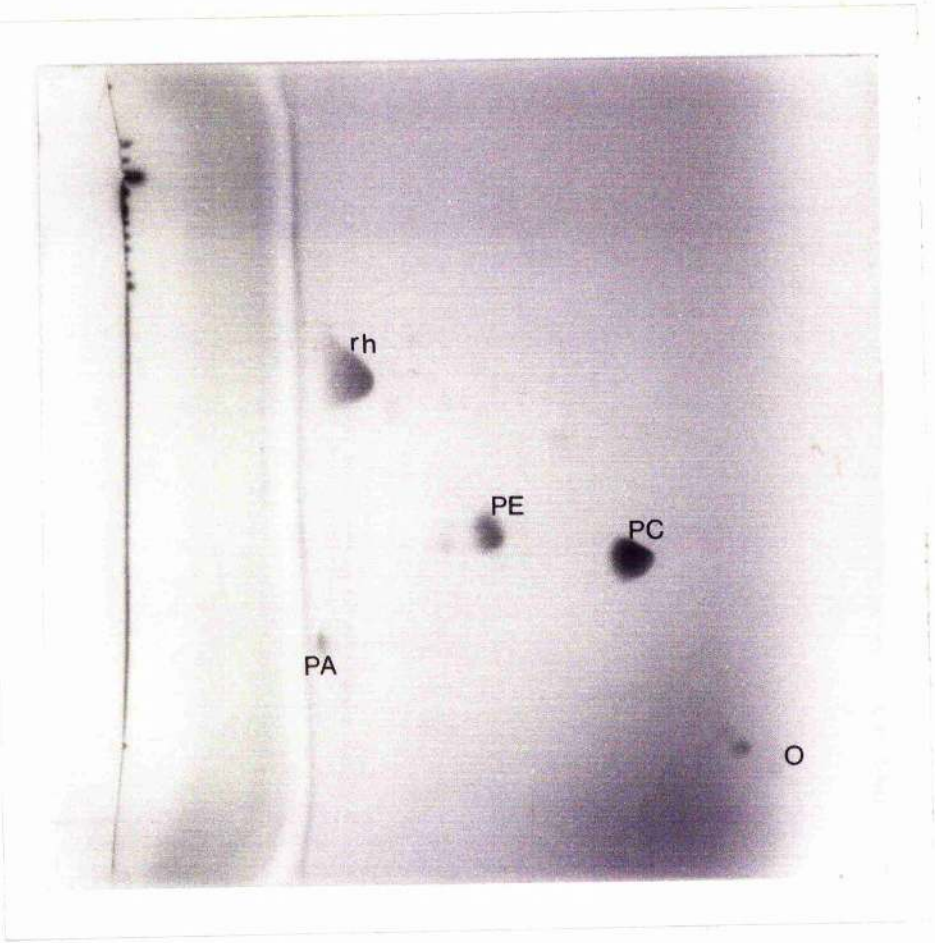


FIGURE 22. Two dimensional TLC of phospholipid fraction of strain *16* grown in N&S medium, IP extraction.

Solvent system: 1. chloroform-methanol-ammonia (25%, w/v)-water. (160:88:11:11, v/v).

2. chloroform-methanol-di-iso butyl ketone-acetic acid-water. (45:15:30:20:4, v/v).

Detection: iodine vapour.

Key to spots: 0 = origin, PC = phosphatidylcholine; PE = phosphatidylethanolamine; PA = phosphatidic acid; rh = rhodamine.

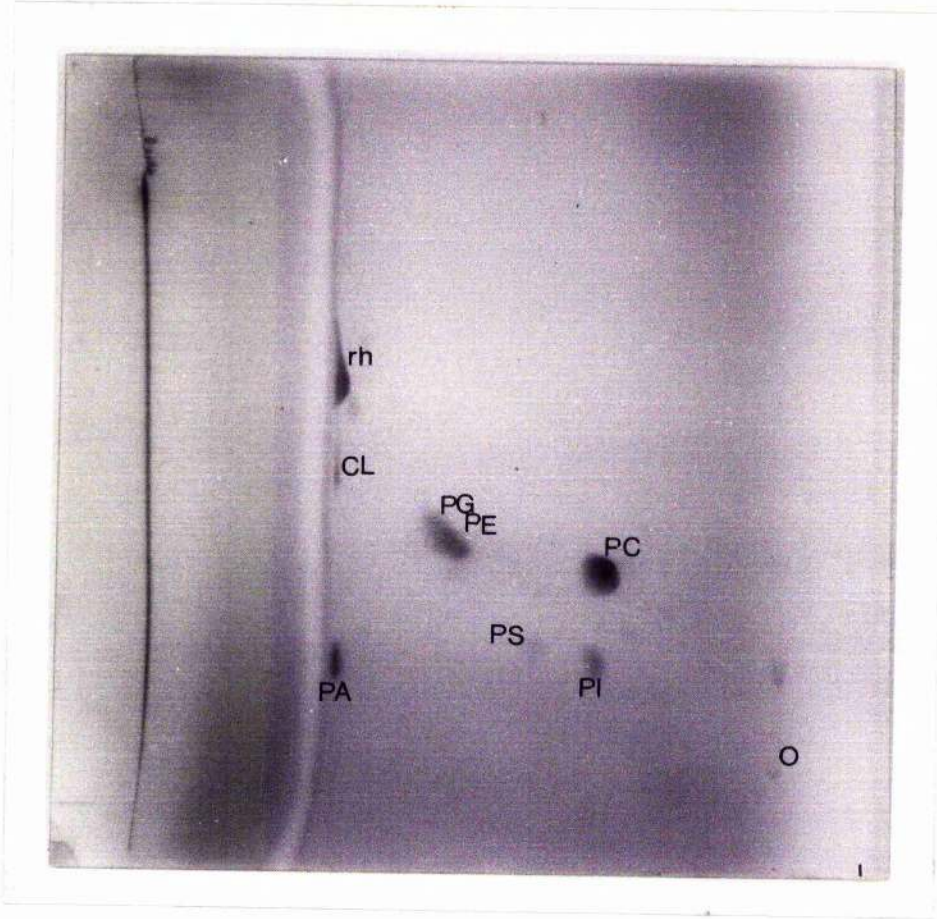


FIGURE 23. Two dimensional TLC of phospholipid fraction of strain *16* grown in N&S medium, CM extraction.

Solvent system: 1. chloroform-methanol-ammonia (25%, w/v)-water. (160:88:11:11, v/v).

2. chloroform-methanol-di iso butyl ketone-acetic acid-water. (45:15:30:20:4, v/v).

Detection: iodine vapour.

Key to spots: 0 = origin, PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PA = phosphatidic acid; CL = cardiolipin; rh = rhodamine.

3.4 Screening of strains for variations in fatty acid composition

Because the quality of the lipid produced by a potentially commercially important strain of fungus is important, in addition to the overall yield, the following aspects of fatty acid composition were screened chromatographically for several of the strains grown in MMM + 3% glucose for 18 days in orbital shaker at 37°C, CM extraction.

3.4.1 Fatty acid composition of total lipid

The fatty acid composition of the total lipid extracted from mutants *MSE*, *ynic 5 ribo 5* and diploids *MSE//ane bio*, *bio met//ane bio*, *ane bio//yw 3 s 12 nic 2*, *bio met//yw 3 s 12 nic 2* was analysed by GLC. The results are shown in Table 21.

As found for the wild type *16*, there are possibly two isomers of 18:2 and, the major fatty acids are 16:0, 18:1 and 18:2, the proportions being variable between the strains. All the diploids assayed contained a high proportion of 18:2, elevating the proportion of unsaturate/saturate to about 20 percent greater than in the two mutants tested.

Another interesting observation is that, the mutant *ynic 5 ribo 5* contained much higher proportions of 16:0 whilst *MSE* showed a predominance of 17:0 compared with the other strains.

Main differences between wild type *16*, mutants and diploids are:

(i) higher 18:0

(ii) lower 18:2 (except *ynic 5 ribo 5*), and

(iii) higher 18:1 in strain *16*.

3.4.2 Fatty acid composition of neutral lipid sub-fractions

Since for any commercial process, the fatty acids of the triglyceride fraction are of primary interest, the total lipid was fractionated into neutral lipid sub-classes by preparative TLC and the fatty acid composition of the fractions determined. The separation obtained is shown in Figure 24.

The band corresponding to sterols (band 1) did not show any fatty

Strains	Fatty acid (% of total)					
	16:0	17:0	18:0	18:1	18:2(?)**	18:2
*16**	21.4	5.2	18.2	20.4	T	34.8
MSE	17.9	22.8	T	13.1	7.9	38.3
ynic 5 ribo 5	38.9	T	8.8	14.2	12.7	25.5
MSE // ane bio	22.1	T	T	12.9	5.5	59.5
bio met // ane bio	23.4	T	T	12.7	8.1	55.8
ane bio // yw 3 s 12 nic 2	19.9	8.5	T	12.2	T	59.4
bio met // yw 3 s 12 nic 2	17.9	5.0	T	10.3	T	66.8

T = traces (<5% of total)

* = data from Table 16

** = possible isomer

TABLE 21. Fatty acid composition of total lipid of haploid and diploid strains of *A. nidulans*.

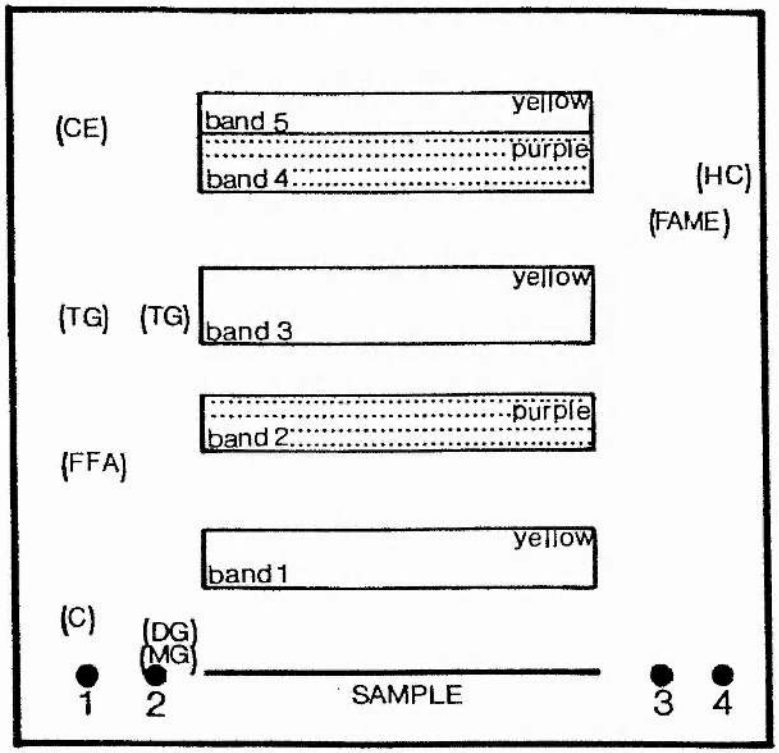


FIGURE 24. Neutral lipid bands of TLC fractionated total lipids of haploid and diploid *A. nidulans* strains.

Key to bands: 1 = sterol; 2 = free fatty acid; 3 = triglyceride; 4 and 5 = sterol ester.

Key to standard 1, 2, 3 and 4: C = cholesterol; FFA = free fatty acid; TG = triglyceride; CE = cholesterol ester; FAME = fatty acid methyl ester; HC = hydrocarbon; MG = monoglyceride; DG = diglyceride.

acid peaks, as expected, whilst bands 2 and 3 contained the components shown in Tables 22 and 23.

Bands 4 and 5 probably correspond to sterol esters and contain only 17:0, as found for wild type *16* (3.2.2.4.3).

Little or no free fatty acid fraction was found for strain *16* after 7 percent hydrated Florisil column chromatography, but a significant band was found for free fatty acid by preparative TLC. This is probably due to the longer time of storage at -15°C before TLC.

Some differences in the fatty acid composition of the triglyceride fractions were found, comparing the wild type, mutants and diploids.

(i) strain *16* exhibits a higher proportion of 18:0 and 18:1

(ii) strain *16* exhibits a lower proportion of 18:2

(iii) diploids show a higher proportion of 18:2

(iv) mutants and dipoids contain higher polyunsaturated and/or long chain fatty acids of unknown identity.

Strain	Fatty acid (% of total)											
	16:0	17:0	18:0	18:1	18:2	20:2	21.6*	22:0	20:5	22:2	22:4	26.2*
ynic 5 ribo 5	27.3	11.5	13.3	7.9	T	-	5.2	6.6	-	-	18.5	9.7
MSE // ane bio	40.3	T	11.8	12.4	11.2	T	9.7	7.5	-	T	7.2	-
bio met // ane bio	22.7	6.5	8.0	11.1	9.4	6.6	-	T	9.2	7.2	19.3	-
bio met // yw 3 s 12 nic 2	44.4	T	15.0	10.8	T	T	7.5	7.6	-	-	14.9	-

* = carbon number, not identified

T = traces (<5%)

- = not detected

TABLE 22. Fatty acid composition of free fatty acid fraction (band 2) of haploid and diploid strains of *A. nidulans*.

Strains	Fatty acid (% of total)							
	16:0	17:0	18:0	18:1	18:2(?)	18:2	21.6*	22:0
*16***	17.4	T	16.2	22.9	T	43.5	T	T
ynic 5 ribo 5	16.8	5.8	7.2	6.7	9.4	45.0	4.7	4.4
MSE // ane bio	16.0	6.9	9.7	10.3	T	52.1	5.0	T
bio met // ane bio	15.6	T	8.8	11.4	T	54.1	10.1	T
bio met // yw 3 s 12 nic 2	20.8	T	11.9	10.5	6.7	34.8	7.8	7.4

* carbon number, not identified

** data from Table 18

T traces (<4% of total)

TABLE 23. Fatty acid composition of triglyceride fraction (band 3) of haploid and diploid strains of *A. nidulans*.

IV. DISCUSSION

The filamentous fungus *A. nidulans* has been widely used in genetic research since the pioneering studies on the parasexual cycle of this mould by Pontecorvo *et al.* (1953).

Fungi are normally haploid organisms, but for a short period of their life cycle they contain two sets of chromosomes. Another striking distinction between fungi and higher organisms is that they exhibit two different kinds of genetic recombination cycle: somatic (parasexual cycle) and sexual.

The parasexual cycle was defined by Pontecorvo (1956) as a sequence of events which consists of: formation of a heterokaryon by the fusion of two mycelial cells of the same species but with genetically dissimilar nuclei, the nuclei of both strains thus becoming intermingled in a common cytoplasm; fusion of dissimilar haploid nuclei; mitotic crossing over and further haploidization. This cycle has the same effect as the sexual cycle, providing genetic recombination and thus increasing the available genetic variation. However, the frequencies of vegetative nuclear fusion, mitotic crossing over and further haploidization have been estimated by Pontecorvo (1956) to occur once in 10^6 or 10^7, once in 500 nuclear divisions and once in 1,000, respectively. These low frequencies mean that the parasexual cycle is of small effect, in natural conditions, in comparison with the sexual cycle in a homothallic sexually reproducing mould like *A. nidulans*. However, it is of great importance for the Deuteromycetes (Fungi Imperfecti) for which no sexual cycle has been observed, and for studies in the laboratory where a great number of crosses can be easily carried out.

In this work, using the technique described by Pontecorvo *et al.* (1953) six auxotrophic mutants were crossed in all possible combinations, followed by isolation, after heterokaryon formation of diploid colonies. All crosses were successful in producing diploid colonies which

characteristically exhibited conidia of larger volume than the haploid parents.

The use of a diallel cross system was based on the work of Baracho and Azevedo (1972), who suggest Griffing's (1956) biometric model of diallel cross analysis for the study of the inheritance of quantitative characters in fungi. Since variation in the lipid content between different strains under the same environmental conditions is due to hereditary factors, it should be possible to estimate and test, through a diallel cross model, the combination abilities and to look for the quantitative effects of the genes which take part in lipid synthesis and accumulation.

The preliminary trial in MM + 3% glucose (Table 6, Figure 3), showed that although yields were low and any differences small, the mutant *ane bio* has the highest cellular lipid content and *MSE* is the best biomass and total lipid producer, compared with the wild types, other mutants and diploids.

The results obtained for total lipid in the diploids tested were shown to be an average of the two mutant parents.

The diploid *ane bio//MSE* (Table 7) gave better lipid accumulation than the three other diploids, when grown unagitated for 11 days in N&S medium. Otherwise, no significant difference was discernible between the crosses investigated.

The use of the parasexual cycle to generate improved strains, with increased capacity to synthesize economically important fermentation products, was first suggested by Pontecorvo and Roper (1952) and Pontecorvo and Sermonti (1953) and has since been widely used (Berry, 1983) as a powerful technique for the selection of improved strains. Its great success is perhaps best exemplified by the improvement of penicillin titre in indústrual strains of *Penicillium chrysogenum* (Alikhanian, 1970).

The number of experiments in this work is rather small and the

results so far obtained are not so significant as to allow a firm conclusion to be drawn. However, the lack of significant improvement in lipid production may be due to:

(i) sub-optimal conditions for lipid accumulation (which will be discussed later) and/or

(ii) the combination of strains used in the crosses.

All crosses were carried out with mutant strains which were derived from a single wild isolate (the mutants came from Glasgow stock, which is derived from a single wild isolate - Clutterbuck (1974)).

The importance of the genealogical relationships of strains was first discussed by Sermonti (1957), who suggested that the penicillin yield from diploids was influenced by the relationships of their initial parent strains. More specifically, that more distant and, consequently, more heterozygotic strains may yield more active diploids than closely related strains.

In contrast, Kameneva's studies (1960) of a large number of diploids formed from biochemical mutants of six *Penicillium chrysogenum* strains of various genealogical relationships, showed that these specific relationships of the initial strains did not influence the productivity of diploids.

MacDonald (1981) also emphasizes that crossing between strains widely divergent in lineage should be avoided, as gross chromosomal damage and a lack of recombination may result, thereby impeding the isolation of progeny with better yields than either parent.

Some valuable conclusions were reached by Merrick and Caten (1973), who carried out a selection program for penicillin-producing strains of *A. nidulans*, applying some of the techniques used when breeding for quantitative characters in higher organisms. They explained the increased productivity as being due to the fact that during the cross program, fixation of different genes had occurred. A further cross

between genetically different selected lines gave a better penicillin titre showing that many genes contribute to antibiotic synthesis by a particular strain and these genes can be recombined in an additive manner.

On the other hand, according to Pardee (1969), the rate of enzyme synthesis in industrially important microorganisms can be improved by increasing the number of structural genes per given cell. Clarke (1973) made a further contribution to this area by reporting that the enzyme yield may be increased by one, or a combination of the following genetic methods:

(i) increase in gene dosage

(ii) mutation in regulator genes concerned with induction and repression controls

(iii) mutations giving decreased catabolite repression

(iv) promotor mutations giving increased rates of gene transcription.

It is interesting to note here that Boulton and Ratledge (1982) found that the presence of the enzyme ATP:citrate lyase is directly correlated to the phenomenon of oleaginicity in yeasts. It was concluded that this enzyme probably acts as the rate-limiting reaction for lipid biosynthesis, since it is required to provide carbon for fatty acid synthesis in the form of acetyl CoA, in the cytosol, from citrate exported from the mitochondria.

All these studies emphasise that the production of lipid could theoretically be enhanced by means of genetic methods. So, if a strain is already known to be oleaginous, and one seeks only quantitative changes in the variants, it is wise to start a selection program, screening a large number of variants of this strain, under a few different fermentation conditions, rather than to screen a small number of variants under a wide variety of environmental conditions (Chang and Elander, 1979).

In addition, undoubtedly, the mutation and selection technique should have considerable scope for manipulation of fatty-acid profiles of triglycerides.

As mentioned previously, environmental conditions can markedly influence the characteristics of lipid accumulation. This can be seen from this work comparing early experiments on lipid production of wild types, mutants and diploids grown in MM + 3% glucose, where lipid content of all strains was low, with the results obtained from later work on wild type, strain *16*, where growth produced much improved yields of biomass and lipid.

In view of these latter findings on the influence of culture conditions on lipid content and yield of *A. nidulans* wild type (strain *16*), the most likely explanation for the failure of the crosses tried here to improve lipid yield would seem to be initial selection of non-optimal culture conditions for the screening experiments. A re-examination of these strains under optimal culture conditions for lipid accumulation might well now prove fruitful in revealing differences and identifying improved strains.

The diagram shown in Figure 25 illustrates the influence of twelve different growth conditions on biomass, lipid content and total lipid of strain *16*, after 8-9 days of incubation. Of the eight different media tried, that which showed best results for lipid accumulation was MMM + 12% glucose + 1% $NaNO_3$. Different media from this had been used in the past by others to attain lipid accumulation in *A. nidulans* (see Introduction), but attempts to use some of these media gave unfavourable results, with the strain used in this work. Strain *16*, grown in the conditions suggested by Garrido *et al.* (1958) (Table 8 and no. 2 in Figure 25), gave a striking improvement in biomass yield, over all other media, but the lipid content remained at the normal low level, around 3-4 percent of dry weight. However, the total lipid produced per unit volume of this medium reached almost 1 mg per ml, being greater than in MM + 3% glucose or N&S medium under the same cultural conditions (unshaken cultures).

Weete (1980), considers an economically viable strain to be one which is able to accumulate 20 percent of its dry matter as lipid.

FIGURE 25. Biomass, lipid content and lipid yield of strain *16*, grown in different media and culture conditions.

	Media	Conditions	Days	Temp. (^{o}C)	C:N (mass ratio)
1.	MM + 3% gl.	shaker	8	37	12:1
2.	GGW	shaker	8	37	73:1
3.	MM (9% gl. gradual increase)	incubator	8	37	36:1
4.	N&S	fermenter	9	30	69:1
5.	N&S	incubator	8	30	69:1
6.	N&S (5% gl.)	incubator	8	30	20:1
7.	MMM (17% gl.)	incubator	8	30	69:1
8.	MMM (12% gl. + 0.1% $NaNO_3$ + 0.5% KH_2PO_4)	incubator	8	30	291:1
9.	MMM (12% gl. + 0.1% $NaNO_3$ + 0.5% KH_2PO_4)	shaker	8	30	291:1
10.	MMM (12% gl. + 0.2% $NaNO_3$ + 0.5% KH_2PO_4)	shaker	8	30	146:1
11.	MMM (12% gl. + 0.1% $NaNO_3$ + 0.5% KH_2PO_4)	fermenter	8	30	291:1
12.	MMM (12% gl. + 0.1% $NaNO_3$)	vortex stirrer	9	37	291:1

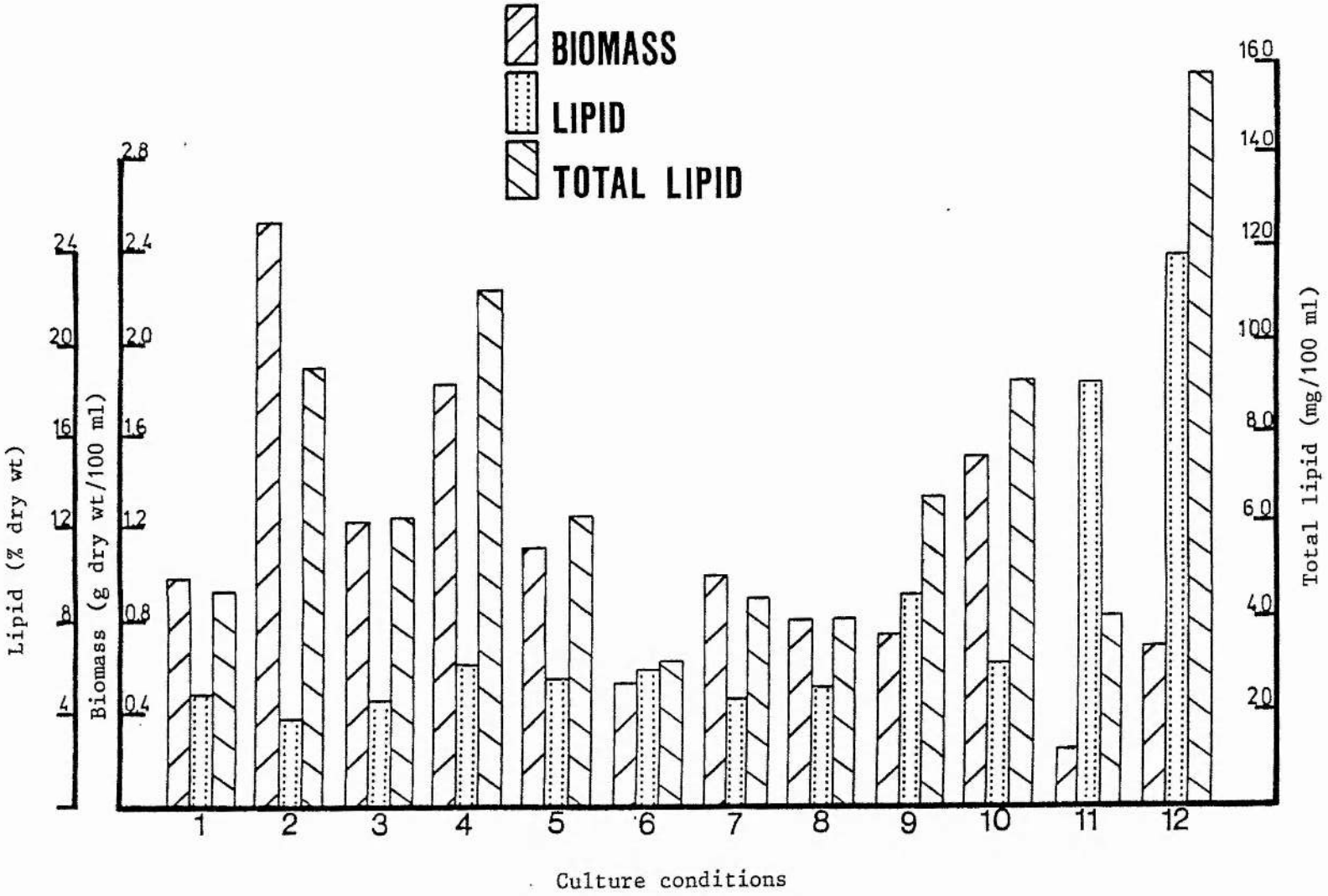

BIOMASS
LIPID
TOTAL LIPID
Lipid (% dry wt)
Biomass (g dry wt/100 ml)
Total lipid (mg/100 ml)
Culture conditions
1
2
3
4
5
6
7
8
9
10
11
12

FIGURE 25.

Ratledge (1982), considers 25 percent (w/w) as the minimal value, unless there is some special attribute for the particular microbial oil.

The main differences between the media tested for lipid accumulation, in strain *16*, were the absolute and relative glucose and sodium nitrate concentrations. An increase in glucose in the medium was accompanied by rapid growth and a large biomass yield (Tables 9 and 11). As expected, lipid accumulation only occurred after nitrogen had expired from the medium. These results are in accordance with the generally accepted view that insufficient nitrogen supply leads to accumulation of lipids in most oleaginous microorganisms growing in batch cultures.

The best carbon:nitrogen ratio (on a mass basis) for lipid accumulation, in strain *16*, under the cultural conditions tested, seems to be 290:1.

However, as can also be seen, biomass and lipid content may be greatly affected by agitation and aeration conditions. When strain *16* was grown in the same medium, but by different cultural methods, the biomass and lipid content increased in the following order: unshaken < shaken < fermenter < vortex stirrer, showing that the aeration rate, or perhaps fungal morphology, play an important role in biomass production and lipid accumulation.

Fungi are normally aerobic organisms and in submerged cultures they must extract their oxygen supply from the medium. Therefore, a constant input and exchange of oxygen with the aqueous solution by aeration and agitation is vital (Berry, 1975). In addition to its requirement as the terminal electron acceptor for aerobic respiration, oxygen is required for the biosynthesis of unsaturated fatty acids and sterols (Weete, 1980).

In addition to gas transfer considerations, a considerable number of changes occur due to the agitation technique, in particular the form of growth of the fungal culture. The morphology of the fungal cells in unshaken, shaken, fermenter and vortex stirrer culture differed considerably, even when other conditions, e.g. medium composition, inoculum, temperature and pH, were the same.

In unshaken conditions, the culture grew as a mat on the medium surface. The nutrient uptake (glucose and sodium nitrate) was slow and, consequently, also the growth rate. In rotatory shaker and fermenter cultures the growth morphology was in the form of pellets. The pellet size was less in the fermenter than in shaken culture.

The vortex stirrer provided very finely divided particles, possibly even single cells, such that it was necessary to use high-speed centrifugation to harvest the biomass (see Material and Methods).

The formation of these small particles is probably due, mainly, to the shear rate (Solomons, 1975) and produced a clearly increased growth rate, biomass and lipid content (see Figure 25).

Vortex culture, therefore, has been shown here to be the most suitable method of cultivation for maximal lipid accumulation in *A. nidulans*, strain *16*.

Perhaps other media also, apart from MMM, will give better results for biomass and lipid yields in the virtex stirrer. It would also be of interest to grow an inoculum in the vortex stirrer for fermenter culture, where a greater amount of medium could be used and, hopefully, the shear rate adjusted to give "single cell" morphology, under conditions of growth which are consistent in essential detail with large scale fermentation processes, currently used for production of other commercially important products.

Although two-thirds of the dry weight of strain *16* came from mycelium grown on surfaces inside the fermenter, rather than in the medium, the problem might be minimised if the fermenter vessel was filled with medium, i.e. wall growth cannot occur if there are no walls! (Ratledge, 1983, personal communication). However, one may then have difficulties with growth occurring in the overflow/vent from the vessel, which is essential if air sparging is used.

The influence of culture technique on lipid accumulation, has also been reported by others. *Aspergillus niger* gave more satisfactory levels of lipid accumulation in shaken culture than in unshaken culture, and comparing both types of submerged culture; shaken and stirred, stirred

culture proved to be better than shaken for lipid accumulation (Woodbine, 1959).

In earlier studies of lipid content of *A. nidulans*, Garrido and Walker (1958) found that this mould accumulated larger amounts of lipid in shaken culture than in a fermenter. Gad *et al.* (1959) also found that the lipid content of this mould could be affected by cultural conditions. Unshaken culture gave better results than shaken, when *A. nidulans* was grown in molasses medium.

Other factors also have been reported to affect lipid content in fungi, such as temperature, pH, and mineral concentrations.

The effect of temperature on lipid accumulation in *A. nidulans* was studied by Garrido and Walker (1953b) and Gregory and Woodbine (1953). It was found that this mould showed a better growth rate at 37°C than at 25°C or 30°C, but a higher lipid content was found at 25-30°C.

In this work, no marked effect on lipid accumulation was observed at growth temperatures of 25, 30 and 37°C.

In his review, Weete (1980) suggests that the temperature has little influence on the lipid content of fungi, but changes in lipid composition, particularly in relation to the saturated /unsaturated ratio, have been found. The degree of unsaturation has been reported to be high at low temperature conditions and vice-versa. However, this influence of growth temperature on lipid composition was not investigated in the present work.

Increased potassium dihydrogen phosphate concentration in the medium appeared not to affect fat content (Tables 13 and 14). Enebo *et al.* (1946) and Pan, Andreason and Kolackov (1949), found that potassium dihydrogen phosphate improved sugar utilization by *Rhodotorula gracilis*, but it also had no effect on lipid accumulation. The highest (0.5 percent, w/v) concentration tried in this work was avoided in subsequent experiments, since it favoured pigment production by the fungi. This pigment was thought to be melanin or melanin-like pigments, which are usually present in

A. nidulans cultures. Rowley and Pirt (1972), studied melanin production by *A. nidulans* in batch culture and found that both forms of melanin, soluble (present in the medium) and insoluble (present in the mycelium), were produced under normal conditions of growth. They also observed that the production of both forms could be activated under conditions of restricted growth rate, such as carbon and nitrogen exhaustion from the medium.

However, there is no evidence from others' work that potassium dihydrogen phosphate affects pigment production.

The presence of these pigments in excess in the medium may have interfered with glucose estimation by DNSA method (Tables 13 and 14). A possible explanation for this interference may be that the pigment exhibited absorbance at the wavelength (540 mn) used, although this was not verified. In addition, this pigment undoubtedly interfered with lipid chromatography and analysis (see 3.2.2.2.2).

Little can be said about the influence of pH in this work. Most of the experiments were carried out at pH 6.5 (it is known that $NaNO_3$ is better absorbed at pH's around 6.0; Berry, 1975); the only exception being with GGW medium (pH = 3.8), where no improvement in lipid content was observed.

One of the objectives of this work was to investigate the lipid composition of *A. nidulans*. The results showed that the method used for extraction and the growth medium composition affected lipid composition, both quantitatively and qualitatively. Although the proportion of each lipid class (neutral, glycolipid and phospholipid), separated on Florisil column chromatography showed no significant difference (Table 15) in either medium, further fractionation of neutral lipid classes on a hydrated Florisil column showed that the proportion of triglycerides and sterols does change. The triglycerides are the major class in N&S medium, but sterols are the major class in MMM (Table 17).

Therefore, it was concluded that the enhanced lipid content in the latter medium (see Figure 25) is accounted for mainly by an increase in the amount of sterols. High sterol content has also been observed in *Saccharomyces cerevisiae* (Clausen *et al.* 1974 and Christiansen, 1978), where about 40-50 percent of the lipid accumulated in the oil droplets was shown to be sterol, either free or esterified. Ratledge (1982), considered this organism as non-oleaginous, despite its production of large amounts of lipid, since the class which is of principal commercial interest is the triglycerides. The microorganisms defined as oleaginous exhibit triglyceride as the major constituent (usually about 80-95 percent) of their lipids.

The medium composition also affected the proportion of sterol esters. In N&S medium, a higher proportion of sterol ester can be seen, compared with MMM on TLC (Figures 12 and 13), which was confirmed gravimetrically by the class fractionation on hydrated Florisil (Table 17).

The polar lipid composition is clearly also influenced by the medium. TLC of phospholipids of cells grown in N&S medium showed PC and PE as major components and PI, PS, PG, CL and PA as minor components. While in MMM, only PC appeared clearly.

Analysis on 2D-TLC of the fractionated polar lipid fraction of the cells grown in MMM in fermenter, could not be carried out, due to bad streaking, possibly caused by the large amount of pigments which were produced in this particular medium.

In addition, looking back to Table 15, it can be seen that there is a lack of recovery of lipid P on MMM, which may indicate novel phospholipids, with a much lower P content than those normally found. Another interesting observation is that the ratio of glycolipid/phospholipid is two-fold higher in MMM than in N&S medium. This increase in sterol and glycolipids and the change in phospholipid composition must result in striking differences in the structure and possibly, function of the cell membranes in the two media.

The basis for the difference in the sterol content in the two media is not understood, but it may have physiological significance, since sterols are known to play an important role in the growth and development of fungi.

A relative preponderance of glycolipids over phospholipids has also been observed in *Aspergillus niger* by Laine *et al.* (1972). It has been suggested that acidic glycolipids can readily replace phospholipids as structural components, both in *A. niger* (Laine *et al.*, 1972) and in bacteria (Minnikin *et al.*, 1971).

It was observed in this work that the amount of DGDG exceeded that of MGDG in N&S medium (Figures 20 and 21). Satisfactory resolution of glycolipids in MMM was not possible due to streaking of TLC. This predominance of DGDG has also been observed to occur in algae and non-photosynthetic tissues of plants (Douce and Joyard, 1980).

The medium composition had only a slight effect on fatty acid composition in *A. nidulans*. In MMM, the glycolipid and phospholipid fractions showed a greater proportion of polyunsaturated and/or long chain fatty acids (some unidentified), when compared with N&S medium, whereas, in the latter, a higher proportion of 18:2 in the neutral, glycolipid and phospholipid fractions was observed instead.

However, these differences could also be due to the different ages and stages of development of the cultures. In general, in other fungi, the main change in lipid composition resulting from increasing age of culture, is a decrease in the degree of saturation in fatty acids (Brennan *et al.*, 1974). However, in this work, this may not be the case as polyunsaturates and/or long chain acids are apparently present in higher proportions in MMM (harvested after 18 days of cultivation), than in N&S medium (harvested after 12 days of cultivation).

It is interesting to note that each lipid class (neutral lipid, phospholipids, glycolipids) and sub-classes of neutral lipids, had different and distinctive fatty acid compositions. The glycolipid fatty acid composition varied with the two media studied, so no generalizations can be made, but the

phospholipid fraction possessed relatively greater amounts of 16:0. The triglyceride fraction contained 18:2 as the major component and the sterol esters, interestingly, exhibited 17:0 as the only fatty acid esterified to sterol.

Margarinic acid (17:0) is always reported as a minor component in fungi and other microorganisms. In addition, plants, yeasts and moulds have been reported to contain any of 16:0, 16:1, 18:1, 18:2 and 18:3 as the major fatty acids, but usually a given lipid class contains several of them.

In yeast, sterol esters 16:1 and 18:1 are the principal fatty acids (Bailey and Parks, 1975). Adams and Parks (1967) found a predominance of 16:1 (about 35 percent) in one strain of *Saccharomyces cerevisiae*, but in another strain of the same species, Pereira *et al.* (1983), found 16:1 and 18:1 (about 40 and 38 percent, respectively), as the principal fatty acids.

There are at least two possible reasons for *A. nidulans* to contain only one fatty acid sterified to sterol:

a) the enzyme responsible for esterification may possess an absolute specificity for 17:0

b) there is a specific acyl donor, which presents 17:0 as the only available fatty acid to the acyl transferase. This, of course, again implies an absolute specificity of the enzyme which synthesises the acyl donor.

It is known that there are three kinds of enzyme that catalyse the esterification of sterols in animals:

(i) a purified pancreatic sterol ester hydrolase, which has both esterification and hydrolytic activities

(ii) acyl-CoA:cholesterol-O-acyl transferase, which has been detected in mitochondria and endoplasmic reticulum of liver

(iii) lecithin:cholesterol acyl transferase (LCAT), which is found

predominantly in plasma and catalyses the transesterification of fatty acids from lecithin (PC) to cholesterol.

The acyl donor for sterol ester biosynthesis in animals, therefore, seems to be either acyl CoA or phosphatidylcholine, whereas, in plants, either PC or diglycerides fulfil this function (Mudd, 1980).

The biosynthesis of sterol ester in cell-free preparations of *Phycomyces blakesleeanus* has been studied by Bartlett *et al.* (1974). It was found that PC appears to be the donor, although some acylation by way of acyl CoA was also measured

In this work 17:0 was also detected in more than trace amounts in neutral-glycerides and phospholipid fractions.

Perhaps there is a physiological explanation for this fatty acid specificity of sterol ester, possibly a structural requirement for a precise function of sterol esters in the cell.

The choice of extraction method undoubtedly affects the lipid composition both quantitatively and qualitatively.

Initial column fractionation of the lipid of strain *16*, extracted by the two methods (Table 15), showed a different lipid class composition. IP gave: neutral lipid = 86 percent; glycolipid = 7 percent; phospholipid = 7 percent, whilst CM gave: neutral lipid = 75 percent; glycolipid = 8 percent; phospholipid = 17 percent.

CM extraction method was shown to extract sterols (both free and esterified) and polar lipids more efficiently than IP (Tables 15 and 17).

The ability of methanol to extract membrane-associated lipids is well known. It disrupts hydrogen bonding and hydrophobic forces between the lipids and the protein, so that the increased levels of the polar lipids and sterols may suggest that either both are bound to membrane protein, or, more likely, that the polar lipids are bound to protein and the sterols are, in turn, bound to polar lipids.

Rose (1981), reported that free sterol and polar lipids are located in membranes, but sterol esters are located with triglycerides in

intracellular low-density vesicles. Also, Christiansen (1979), isolated and analysed the lipid droplets of *Saccharomyces cerevisiae* and found a composition of 95 percent lipid and 5 percent protein. The lipid fraction contained 45-50 percent triglycerols and 45-50 percent sterol esters. If sterol esters are found in similar sites in *A. nidulans* it is surprising that an improved yield of these are obtained with CM, since one would expect IP to be just as good as a method for extraction of free, unbound lipid.

From Figures 22 and 23, it is apparent that the phospholipid composition was also affected by the extraction method. With CM extraction, PI, PS, PC, PE, PG, CL and PA were detected, whilst with isopropanol only PC, PE and PA were found.

A possible cause for the apparent lower efficiency of IP in the extracting phospholipid classes is the multiple aqueous washes used in the IP method. Glycolipids may be lost in the aqueous wash, as may the acidic phospholipids, such as PS, PI and PA (Nelson, 1972).

Another significant difference between the two extraction methods, seen in Table 20, is the very large amount of PA found after CM extraction, but not after IP. This almost certainly suggests the action of a phospholipase D. This enzyme, confined to the plant kingdom, hydrolyses phospholipids with the consequent formation of phosphatidic acid. The data in Table 20 are also strongly suggestive of the fact that PC was the principal phospholipid attacked by this enzyme since the proportion of this phospholipid class was also reduced after CM.

The presence of phospholipases in fungi, including phospholipase D, has also been reported by Blain *et al.* (1978).

It is known that the activity of phospholipase D is stimulated by solvents such as chloroform and ether, whilst hot isopropanol is known to inactivate this enzyme (Kates, 1982).

The fatty acid composition of fractionated and unfractionated

lipids does differ slightly between the two extraction methods. In particular, the total and neutral lipids which show a higher proportion of 17:0 after CM, as compared with IP, extraction.

The lipid composition of some other filamentous fungi, compared with strain *16* in the two different media and after extraction by the two methods studied, are given in Table 24. As can be seen from this table, there is wide species variation in lipid composition. Also apparent from this work, is the profound influence of both medium composition and extraction method, which makes comparison between different laboratories difficult. However, with the exception of the high proportion of sterol in MMM, the lipid composition of *A. nidulans* reported here, lies within the range exhibited by the three other species listed in Table 24.

The studies presented here, particularly with regard to the variable sterol content of *A. nidulans*, underline the importance of ascertaining the precise lipid composition of an "oleaginous" microorganism before considering its adoption for an industrial process. It is clear that increased total lipid content as a result of manipulation of medium composition, may be as a result, largely or solely, of the accumulation of an undesirable/less desirable lipid component.

Finally, a better overall recovery of lipids was achieved with the CM extraction method than with isopropanol, although some lipase activity was observed in the former method.

The fatty acid composition of fractionated and unfractionated lipids varied with the strain of mutants and diploids of *A. nidulans*. For total lipid, the proportion of 18:2 was higher in the diploids, making the unsaturated/saturated ratio about 20 percent greater than in the three haploids assayed.

The composition of the neutral lipid sub-classes, fractionated by preparative TLC, showed 5 major bands. The fatty acid composition of these bands for the mutant strains was compared with strain *16*. As for strain *16*, the major fatty acids were: 16:0, 17:0, 18:0 18:1 and

TABLE 24. Lipid fraction composition of some fungi.

Organism		Lipid fraction (% of total)						
		TG	S	SE	FFA	PL	GL	others
*Fusarium oxysporum**		66.8	11.4	11.8	11.4	7.9	-	-
*Penicillium lilacium**		92.3	4.3		3.4	4.6	-	-
*Rhizopus arrhizus**		22.1	16.7		11.7	44.4	-	5.1 (1)
MMM strain *16*	IP	24.3	45.1	5.7	-	7.7	5.2	11.8 (2)
	CM	22.2	36.9	6.3	-	15.1	8.9	10.6 (2)
N&S	IP	74.8	6.2	2.4	-	6.6	7.7	2.3 (2)
	CM	47.3	12.5	8.6	-	19.2	7.0	5.3 (2)

* Ratledge (1982)

Abbreviations: TG = triglyceride; S = sterol; SE = sterol ester; FFA = free fatty acids; PL = phospholipids; GL = glycolipids

others: (1) = fatty acid methyl esters, mono- + diglycerides

(2) = mono- + diglycerides + FFA

possibly two isomeric forms of 18:2.

As expected in moulds, 18:2 is the predominant fatty acid in the triglycerides.

Two bands were visible in the sterol ester region of the chromatogram, a large one, purple in colour and, above that, a thinner, yellow band (under U.V. light, after rhodamine).

Both bands contained 17:0 as the only fatty acid (as found for strain *16*), and were tentatively identified by comparison with authentic standards and specific straining as sterol esters. The basis for their resolution is therefore likely to be due to different sterol moieties.

Table 25 shows the fatty acid composition of some filamentous fungi, compared with the *A. nidulans* strains studied in this work.

As for lipid classes, there is considerable species variation in fatty acid composition, although the differences are quantitative rather than qualitative. There is also marked inter-strain variation in *A. nidulans*, with some strains showing much higher levels of 18:2 than the other species of filamentous fungi listed.

In conclusion, this study has indicated several avenues worthy of further exploration:-

(i) from the promising results obtained with the vortex-stirrer, it would seem likely that lipid content of *A. nidulans* would be improved even further by manipulation of culture conditions. For example, it would be of interest, as already suggested, to use a vortex-stirrer grown inoculum for a fermenter culture, grown under conditions which minimise wall growth.

(ii) having completely optimised growth conditions for lipid accumulation, a re-examination of the mutants, diploids and heterokaryons for improved lipid yield should be undertaken. Other methods of strains improvement and selection could also then be tried, with particular emphasis on lipid quality as some of the strains analysed in this work looked as though they

TABLE 25. Fatty acid composition of total lipid of some fungi.

Organism	Fatty acids (% of total)								
	14:0	16:0	16:1	17:0	18:0	18:1	18:2**	18:2	18:3
*Aspergillus niger**	2	22	3	-	5	7	-	46	11
*Fusarium moniliforme**	1	14	-	-	11	30	-	42	1
*Malbranchea pulchella**	-	11	-	11	27	51	-	-	-
*Mucor globosus**	8	26	8	7	26	8	-	16	-
*Penicillium soppi**	2	41	1	T	8	12	-	32	3
strain *16*	-	26	-	11	17	16	T	30	-
MSE	-	18	-	23	T	13	8	38	-
ynic 5 ribo 5	-	39	-	T	8.8	14	13	26	-
MSE//ane bio	-	22	-	T	T	13	6	60	-
ane bio//bio met	-	23	-	T	T	13	8	56	-
ane bio//yw 3 s 12 nic 2	-	20	-	8	T	12	T	59	-
bio met//yw 3 s 12 nic s	-	18	-	5	T	10	-	67	-

T = traces

* Ratledge (1978)

** 18:2 isomer

were capable of high yields of 18:2.

(iii) of biochemical interest, would be confirmation of the structure and fatty acid composition of the sterol ester fraction, followed by investigation of the enzymology of the acylation and the possible function of the sterol esters.

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